Impact of the Catabolite Control Protein CcpA in *Staphylococcus aureus*

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von

Kati Seidl

von

Wattwil SG

Promotionskomittee

Prof. Dr. Leo Eberl (Vorsitz)
Prof. Dr. Brigitte Berger-Bächi (Leitung der Dissertation)

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Summary

The catabolite control protein CcpA is the main regulator of carbon catabolite repression and activation in low-GC Gram-positive bacteria. In the presence of glucose, CcpA regulates gene expression by binding to so-called catabolite responsive elements (*cre*) located upstream of, or within the promoter regions of the regulated genes. CcpA has been extensively studied in different bacteria, and was found to not only regulate metabolic genes but also to control virulence and antimicrobial resistance. In this study, we analysed the role of CcpA in the pathogen *Staphylococcus aureus*.

Transcriptome analyses showed that more than 150 genes in the exponential growth phase were regulated by glucose in a CcpA-dependent manner, and that even in the absence of glucose, CcpA regulated more than 200 genes. Many of the regulated genes encode metabolic enzymes, suggesting that CcpA plays a similar role in *S. aureus* as it does in other bacteria. However, we also found some aspects of the CcpA regulon, which were specific for *S. aureus*.

The *S. aureus* CcpA was also involved in the regulation of some important virulence factors. Glucose-dependent repression of *hla-*, *spa-,* and *tst*-expression, coding for α-hemolysin, protein A, and toxic shock syndrome toxin, respectively, was dependent on CcpA. The presence of putative *cre*-sites in the promoter regions of these genes suggested that they were under direct control of CcpA. In contrast, the glucose-mediated and CcpA-dependent downregulation of the capsule genes (*cap*) was probably indirect. Deletion *ccpA* reduced the transcription of *RNAIII*, which is the effector molecule of the *agr* operon and regulates the expression of many exoproteins and cell wall associated proteins. The deletion was also found to significantly reduce methicillin resistance and a Δ*ccpA* mutant was significantly more susceptible to teicoplanin.

Moreover, deletion of *ccpA* abolished the capacity to form a biofilm under static and flow conditions, and was linked to decreased expression of *icaA*, a gene responsible for the expression of PIA, an important component of the *S. aureus* biofilm matrix. Repression of *icaA* was presumably achieved via the upregulation of the TCA cycle genes *citB* and *citZ* in the Δ*ccpA* mutant. Loss of biofilm formation in the mutant also correlated with decreased *cidA* transcription, recently reported to contribute to the production of extracellular DNA, which is a further component of the *S. aureus* biofilm matrix.

The finding that *S. aureus* virulence and carbohydrate utilization are directly linked through CcpA shows an important mechanism, by which this pathogen modulates virulence factor production in response to changes in its environment.

Zusammenfassung

In Gram-positiven Bakterien wird Kohlenstoff-Katabolitrepression hauptsächlich durch das Katabolitenkontrollprotein CcpA bewirkt. In Anwesenheit von Glukose bindet CcpA an spezielle DNA-Sequenzen, ober- oder unterhalb des Transkriptionsstartes bestimmter Gene und reguliert somit deren Expression. Umfangreiche Untersuchungen in verschiedenen Gram-positiven Bakterien haben gezeigt, dass CcpA nicht nur Gene des Stoffwechsels reguliert, sondern auch an der Regulation von Virulenz und Antibiotikaresistenz beteiligt ist. Diese Studie untersucht erstmals die Rolle von CcpA im pathogenen Bakterium *Staphylococcus aureus*.

Eine Transkriptomanalyse zeigte, dass CcpA in Abhängigkeit von Glukose über 150 Gene reguliert. Interessanterweise wurden bereits ohne Glucose über 200 Gene durch CcpA kontrolliert. Die meisten regulierten Gene kodieren Enzyme des Stoffwechsels, was darauf hindeutet, dass CcpA in *S. aureus* eine ähnliche Rolle spielt wie in anderen Gram-positiven Bakterien.

Zudem konnte nachgewiesen werden, dass CcpA die Expression wichtiger Virulenzfaktoren von *S. aureus* kontrolliert. So war die durch Glukose induzierte Repression der *hla, spa* und *tst*-Gene, welche für α-Hämolysin, Protein A und Toxic Shock Syndrome Toxin kodieren, abhängig von CcpA. Die Anwesenheit potentieller CcpA-Bindestellen in den Promoterregionen dieser Gene weist auf direkte Kontrolle durch CcpA hin. Im Gegensatz dazu scheint die durch Glukose induzierte und von CcpA abhänginge Repression der Kapselgene indirekt zu sein. Die Inaktivierung des *ccpA*-Genes führte ausserdem zu einer signifikanten Reduktion der Transkription von *RNAIII,* dem Effektormolekül des *agr*-operons welches die Expression vieler Exoproteine und zellwandassoziierter Protein kontrolliert. Auch verringerte die Deletion von *ccpA* die Resistenzhöhe gegenüber Methicillin und Teicoplanin.

Ferner reduzierte die Inaktivierung von *ccpA* die Fähigkeit von *S. aureus* einen Biofilm zu bilden und konnte mit reduzierter *icaA*-Expression in Verbindung gebracht werden. *IcaA* ist Teil des *ica*-Operons, welches für die Bildung von PIA verantwortlich ist, einer wichtigen Komponente des *S. aureus* Biofilms. Die beobachtete *icaA*-Repression und auch die verminderte PIA-Bildung in der Mutante wurden wahrscheinlich über die Hochregulierung der Krebszyklusgene *citZ* und *citB* bewirkt. Ausserdem korrelierte die verminderte Biofilmbildung der Mutante mit reduzierter Transkription von *cidA*, einem Gen welches an der Produktion extrazellulärer DNA (eDNA) beteiligt ist. eDNA ist ein weiterer Bestandteil der *S. aureus* Biofilmmatrix.

S. aureus CcpA stellt somit ein wichtiges Bindeglied zwischen Virulenz und Metabolismus dar, welches es dem Bakterium ermöglicht die Produktion wichtiger Virulenzfaktoren auf die gegebenen Bedingungen in seiner Umgebung abzustimmen.

1 Introduction

1.1 Staphylococcus aureus

Staphylococcus aureus is a factultative anaerobic, Gram-positive bacterium belonging to the family of *Micrococcaceae*. It may appear as a commensal on human skin and colonizes up to 30 % of the healthy population, usually in the nares (4). However, it is capable of producing a wide array of virulence factors and therefore it commonly causes a variety of diseases ranging from superficial lesions to systemic and life-threatening infections such as osteomyelitis, endocarditis, pneumonia and septicaemia. The difficulties in fighting this pathogen are the increase in resistance to antibacterial agents together with its increasing prevalence as a nosocomial pathogen (4). The bacterium can adapt to new conditions very quickly, because it is able to aquire new genetic information through gene exchange with other bacteria (117).

1.1.1 Virulence

Archer (4) proposed five stages for the pathogenesis of *S. aureus*: colonization, local infection, systemic dissemination and/or sepsis, metastatic infection and toxinosis. At each of the different stages, *S. aureus* encounters the immune response of the host. However, *S. aureus* is able to produce a wide array of virulence factors, which specifically influence the host's function and immune response to allow the pathogen to successfully survive in the host. The main mechanisms the pathogen uses to overcome immune responses are (41):

1. Inhibition of neutrophil chemotaxis
2. Killing of leukocytes
3. Resistance to phagocytosis
4. Resistance to killing by antimicrobial peptides
5. Resistance to killing by lysozyme
6. Ability to survive in neutrophil phagosomes
7. Production of immunomodulatory molecules
8. Subversion of the humoral immune response

1. Inhibition of neutrophil chemotaxis

When the bacterium starts to grow in the host, chemoattractants are liberated and are recognized by neutrophils at very low concentrations. Stimulation of the neutrophils activates intracellular signalling cascades, resulting in the migration of more neutrophils to the site of infection. The chemotaxis inhibitory protein of staphylococci CHIPS blocks the receptors for the chemotactic signals on the neutrophils and thereby prevents the recruitment of more neutrophils (30). The extracellular adherence protein Eap blocks the intercellular adhesion

molecule-1 on the surface of endothelial cells and thereby prevents leukocyte adhesion, diapedesis and extravasation (20).

2. Killing of leukocytes

Cytolytic toxins produced by *S. aureus* form pores in the cytoplasmic membranes of leukocytes and/or erythrocytes and finally lead to lysis. These toxins comprise α-toxin (Hla), and the bicomponent leukotoxins with γ-hemolysin (Hlg), the Panton-Valentine leukocidin (PVL), leukocidin E/D and leudkocidin M/F-PV-like (104).

3. Resistance to phagoytosis

Protein A (encoded by *spa*), a protein that is anchored in the cell wall of *S. aureus*, binds immunoglobulin G molecules the wrong way round by a non-immune mechanism. This disrupts opsonization and phagocytosis (103, 138). The expression of clumping factor A (ClfA), a fibrinogen-binding protein, was shown to protect *S. aureus* from phagocytosis (102). However, fibrinogen seems not to be necessary for the protective properties of ClfA and the exact mechanism by which ClfA protects *S. aureus* from phagocytosis is not fully understood (101). Microcapsules, which are produced by most clinical *S. aureus* isolates, were shown to reduce the uptake by neutrophils (130). In addition, *S. aureus* has the ability to inactivate the complement system by several mechanisms. The *Stahylococcus* complement inhibitor (SCIN), the extracellular fibrinogen-binding protein Efb and staphylokinase (Sak) inactivate different factors of the complement and in this way block opsonization and/or phagocytosis by neutrophils (75, 112, 113).

4. Resistance to killing by antimicrobial peptides

Resistance to killing by antimicrobial peptides inside a neutrophil by *S. aureus* is achieved through natural modifications of the cell surface. Dlt modifies wall teichoic acid and lipoteichoic acid in a way that the negative charge of the cell surface is partially neutralized by D-Ala esterification and the cell is thus less attractive to cationic molecules (107). MprF modifies phosphatidylglycerol to lysylphosphatidylglycerol and thereby increases the positive charge and also reduces the affinity towards antimicrobial peptides (106). *S. aureus* is also able to secrete proteins that neutralize cationic peptides. For example, binding of staphylokinase to the defensins abolishes their bactericidal properties and the extracellular metalloprotease aureolysin cleaves and inactivates a human defensin (64, 126).

5. Resistance to killing by lysozyme

Lysozyme is a muramidase that cleaves peptidoglycan and is produced by various tissues of the host. It is present in body fluids, such as serum, saliva, sweat and tears and its titre is increased by infection. An increase in lysozyme concentration in blood is an indicator for monocyte and granulocyte proliferation. Lysozyme is therefore an important compound of the innate immune defence of the host. *S. aureus* is completely insensitive to lysozyme. Due to *O*-acetylation of the muramic acid of the peptidoglycan through a membrane bound *O*-acetyltransferase (8). This mechanism greatly contributes to the persistence and success of *S. aureus* in colonizing humans and animals.

6. Ability to survive in neutrophil phagosomes

Once *S. aureus* is engulfed by phagocytosis, it has to face superoxide radicals which are produced by the neutrophils. *S. aureus* has several mechanisms to avoid the lethal effects of these free oxygen radicals, such as the production of carotenoid pigments, which give *S. aureus* its golden colour (77). Other oxidative stress resistance enzymes of *S. aureus* comprise superoxide dismutase, catalase (KatA), alkyl hydroperoxide reductase (AhpC), thiol-dependent peroxidase (Bcp), and thioredoxin reductase (TrxB) (27, 66).

7. Production of immunomodulatory molecules

Furthermore, *S. aureus* is able to express toxins which act as superantigens (SAgs). SAgs, unlike conventional peptide antigens, bind to invariant regions of the major histocompatibility complex (MHC) class II molecules at the surface of antigen-presenting cells outside of the classical antigen-binding groove and also to invariant regions of the T-cell receptor (Fig. 1) (108). This leads to activation of T-cells at orders of magnitude above antigen-specific activation, resulting in a massive cytokine release, which in turn leads to capillary leakage and is believed to be responsible for hypotension, shock, and finally death. Staphylococcal superantigens comprise the toxic shock syndrome toxin (TSST-1), exfoliatins A and B, and various staphylococcal enterotoxins (SEs).

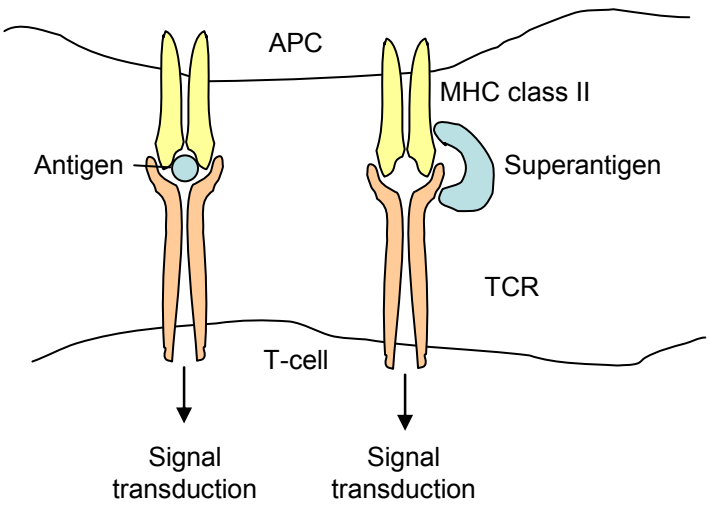

Figure 1: Mechanism of action of superantigens. Left: Normal antigens bind to specific sites of the MHC class II molecules of antigen-presenting cells (APC) and thereby lead to the activation of T-cells. Right: Unspecific binding of superantigens to the MHC class II molecule and also to an invariant region of the T-cell-receptor (TCR) leads to the activation of massive amounts of T-cells (adapted from (41)).

8. Subversion of the humoral immune response

By binding to platelets and thereby stimulating their activation, *S. aureus* can subverse the humoral immune response of the host. Platelet activation is achieved by fibronectin binding protein A or ClfA or B (55, 87, 125). Contact is made with the platelet through a bridge of fibronectin or fibrinogen which bind to *S. aureus* binding proteins on the one hand and to platelet receptors (integrins) on the other hand. Platelet activation requires the binding of antibodies to ClfA/B or fibronectin-binding proteins. These antibodies are found in almost all humans at low levels and can be exploited by the bacterium. *S. aureus* can thus grow in platelet-fibrin thrombi and thereby escape the attention of neutrophils. This is probably an important virulence mechanism in the pathogenesis of infective endocarditis (125).

In addition to the factors, which overcome the host's immune response, *S. aureus* also produces various enzymes such as proteases, nucleases, lipases, and hyaluronate lyase, which allow the invasion of the host tissue. Host fatty acids, which act as detergents and thereby interfere with bacterial growth, are overcome by fatty acid modifying enzymes (FAME) (94). Binding proteins for fibrinogen, fibronectin, laminin, collagen, vitronectin and thrombospondin promote attachment to endothelial cells and the basement membrane (43). Specific toxinosis is caused by enterotoxins or the exfoliative toxin which causes the staphylococcal scalded skin syndrome (SSSS) (4). Figure 2 summarizes the virulence factors of *S. aureus*.

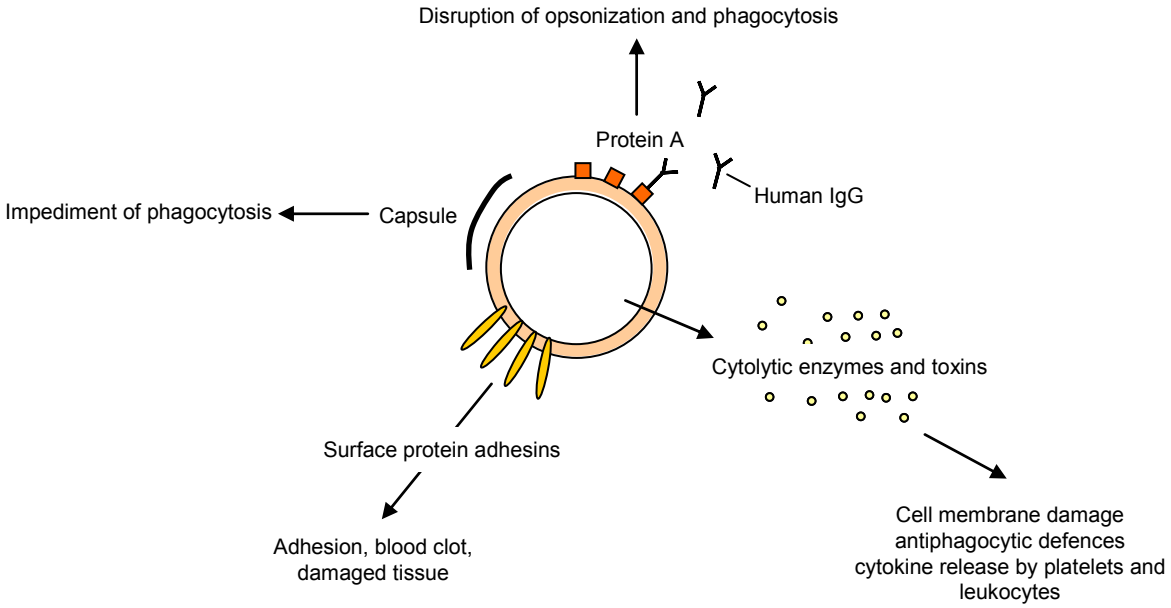

Figure 2: Overview of the *S. aureus* virulence factors (adapted from (41, 42)).

The virulence factors of *S. aureus* contribute to a wide array of infections. The bacterium is the most common agent for furuncles/carbuncles, impetigo, surgical wound infections, pyomyositis (infection of skeletal muscles), botryomycosis (chronic granulomatous infection of skin or viscera), acute/right-sided endocarditis, epidural abscesses, toxic shock syndrome and scalded skin syndrome. But also cellulitis, hospital acquired bacteraemia, hematogenous osteomyelitis, septic arthritis, brain abscesses, hospital-acquired pneumonia, empyema (pus and fluid in the body cavity), septic shock, food-borne gastroenteritis and renal carbuncles are caused by this pathogen (4). Because some of the virulence factors of *S. aureus* are located on mobile genetic elements such as pathogenicity islands (e.g. TSST-1) or lysogenic bacteriophages (e.g. PVL), they can easily spread by horizontal gene transfer (42).

1.1.2 Antibiotic resistance

Since the discovery of the β-lactam penicillin by Alexander Flemming in 1928, many new antibiotics have been developed. However, their introduction was always accompanied by a rapid appearance of resistant bacteria (145). Resistance mechanisms are all based on either inactivation of the antibiotic, prevention of the antibiotic to reach its target, or alteration of the target of the antibiotic by mutation, modification or overexpression (10). All these mechanisms emerge either through the mutation of existing genes or by the acquisition of new resistance determinants. The fact that bacteria occur in high numbers, have short generation times and possess efficient mechanisms for gene exchange favours the rapid evolution of resistance or even multi resistance. Table 1 summarizes the most common antibiotics, their mode of action and resistance mechanisms in *S. aureus*.

Table 1: Main antibacterial classes, mechanisms of action and resistance mechanisms.

Antibacterial class (examples)	Principal target	Resistance mechanisms in *S. aureus*
Aminoglycosides (Kanamycin, Gentamicin)	Protein synthesis: initiation complex/translation	Aminoglycoside modifying enzymes (141)
β-lactams (Penicillin, Methicillin)	Cell wall synthesis: transpeptidation of peptidoglycan	β-lactamases, low-affinity PBP 2a, PBP alteration (mutation, overexpression) (141)
Chloramphenicol	Protein synthesis: peptidyl transferase	Chloramphenicol acetyltransferase, efflux, multidrug transporters (119)
Fusidic acid	Protein synthesis: elongation factor G	Chromosomal mutations, impermeability, inactivation, efflux (136)
Glycopeptides (Vancomycin, Teicoplanin)	Cell wall synthesis: transpeptidation and transglycosylation of peptidoglycan	D-alanyl-D-lactate-containing peptidoglycan precursors (*vanA*), higher cell wall D-ala-D-ala content (10, 141)
Macrolides, lincosamides, streptogramins B and ketolides (Erythromycin, Clindamycin)	Protein synthesis: 50S ribosome	23S rRNA methylase, efflux, macrolide phosphotransferase, lincosamide nucleotidyltransferase, acetyltransferase (38, 73, 141)
Mupirocin	Protein synthesis: isoleucyl-tRNA synthetase	Acquired isoleuyl-tRNA synthetase, chromosomal mutations *ileS* (39)
Oxazolidinones (Linezolid)	Protein synthesis: 50S ribosome	Mutational alterations of 23S rRNA gene (141)
Quinolones (Ciprofloxacin)	Nucleic acid synthesis: DNA gyrase or topoisomerase IV	Altered gyrase, topoisomerase IV, efflux (58)
Rifampicin	Nucleic acid synthesis: RNA polymerase	Chromosomal mutations in *rpoB* (95)
Sulfonamindes/ Trimethoprim (Sulfisoxazol, Sulfamethoxazole, Trimethoprim)	Folate metabolism: depletion of folate pool	Chromosomal mutations, acquired dihydrofolate reductase (114)
Tetracyclines (Tetracycline)	Protein synthesis: ribosomal A site	Efflux, ribosomal protection (25, 141)

The most widely used antibiotics are the β-lactams, which inhibit biosynthesis of the peptidoglycan, the main component of the cell wall. The major subunit of the peptidoglycan is N-acetylglucosamine-*β*-1,4-*N*-acetylmuramyl-pentapeptide, a disaccharide with a stem peptide. These units are crosslinked by transpeptidation and transglycosylation, which is achieved through the so-called penicillin-binding proteins (PBPs). β-lactam antibiotics are structural analogues of the terminal D-alanyl-D-alanine of the stem peptide. Therefore, they

are recognized by the PBPs, bind and thereby inactivate their transpeptidation domains. However, already in the early 1950s, *S. aureus* treatment with penicillin was threatened through the emergence of penicillin resistant strains. These strains express β-lactamases, which inactivate β-lactams via hydrolysis. Methicillin, a semisynthetic β-lactam, is resistant to β-lactamases and was introduced in the 1960s. However, only shortly afterwards, methicillin resistance was reported (63). Methicillin resistance in staphylococci is mainly mediated by the methicillin resistance determinant *mecA*, that codes for a low-affinity PBP, called PBP 2a (137). This PBP allows peptidoglycan biosynthesis in the presence of β-lactams (85). However, methicillin resistance is not only dependent on *mecA* expression, but also on a whole series of chromosomally encoded factors (11, 31). These factors were initially termed *fem* (factors essential for methicillin resistance) (9) or *aux* (auxiliary) factors (31). They are present in resistant and susceptible strains and their inactivation has different impacts on methicillin resistance, ranging from complete hypersusceptibility down to lesser effects.
Today, two categories of methicillin-resistant *S. aureus* (MRSA) are recognized. Hospital-acquired MRSA (HA-MRSA) are the leading cause of nosocomial infections worldwide, especially in intensive care units. Probably because of their continuous exposure to antibiotics in hospitals, they have become multiresistant. They were found to carry superantigenic exotoxins and staphylococcal enterotoxin A as major virulence factors (133). Community-acquired MRSA (CA-MRSA) are a class of MRSA causing infections in communities, where patients have not been exposed to classical risk factors, meaning that these organisms were not acquired in the hospital (2). This class appeared more recently and was found to carry several enterotoxins and PVL, which are probably responsible for the high mortality rate in previously healthy persons (2, 133).

Glycopeptides, such as vancomycin and teicoplanin, comprise another group of antibiotics acting on cell wall synthesis, and play a key role in the treatment of MRSA. As β-lactams, they block transpeptidases, but by a different mechanism. By binding to the D-alanyl-D-alanine of the stem peptide, they block the substrate and prevent it from reacting with transpeptidases or transglycosylases. However, first cases of glycopeptide-intermediate resistant *S. aureus* strains were reported in 1996 and soon afterwards, the first vancomycin-resistant *S. aureus* (VRSA) strains appeared (56). Vancomycin-resistant *S. aureus* (VRSA) strains carry the *vanA* gene cluster, which was most probably acquired by horizontal gene transfer from *Enterococci* (24). *VanA* enables the bacterium to produce altered peptidoglycan pentapeptide precursors. Instead of the terminal D-alanyl-D-alanine, they produce D-alanyl-D-lactate, which leads to a decreased binding affinity for glycopeptides (24). The resistance-mechanisms of glycopeptide-intermediate *S. aureus* (GISA) are not clearly understood, yet. However, at least two or more mutations are required for intermediate resistance. GISA

strains have usually a thickened cell wall, and contain more D-alanyl-D-alanine in the peptidoglycan due to reduced cross-linking (146).
The rapid emergence of resistance to antibiotics requires an acute awareness of physicians. Only a few classes of novel antibiotics have been introduced in the past 40 years. Therefore it is important to avoid the unnecessary use of antibiotics, which might further select for resistant bacteria (53).

1.1.3 Global regulators

S. aureus virulence and resistance are tightly controlled. Regulation takes mostly place at the transcriptional level due to changes in environmental factors such as bacterial density, the presence of antibiotics, pH or CO_2. Each of these factors activate different regulatory systems, which constitute numerous complex networks.

Two-component systems

Two-component regulatory systems (TCS) consist of a sensor histidine kinase and a response regulator protein. They are very sensitive to environmental signals because the sensor either directly binds to a specific extracellular ligand or is associated with a receptor. Upon binding, autophosphorylation of the sensor kinase occurs, meaning that a phosphate residue of an ATP is transferred to the cytoplasmic domain of the sensor kinase. The phosphate is then transferred to the response regulator and the phosphorylation cascade finally ends with the binding of the response regulator to specific DNA sequences, which leads to changes in transcription (16).

Probably the most extensively studied two-component system of *S. aureus* is the accessory gene regulator *agr* (37, 105). Quorum sensing via the *agr*-system has been assigned a central role in the pathogenesis of *S. aureus*. The gene products of the *agr* locus allow the bacteria to determine the actual density of a population and to regulate the transcription of a wide range of genes in response to this density. The actual effector is a RNA molecule, *RNAIII*, which affects virulence gene expression. The *agr* locus is composed of two divergent transcripts, *RNAII* and *RNAIII* which are under the control of the promoters P2 and P3, respectively. In an early growth phase, the expression of *RNAII* is induced through the global regulator SarA. *RNAII* encodes AgrB, D, C and A. The signal molecule AgrD is processed and secreted by the transporter AgrB and thereby becomes the so-called auto-inducing protein (AIP). With increasing density of the population, the signal molecule accumulates in the environment and is therefore more likely to bind to the receptor protein AgrC. This binding leads to phophorylation of AgrC which then activates the signal transducer AgrA. When phosphorylated, AgrA binds to the P2 and P3 promoters and stimulates massive transcription of *RNAII* and *RNAIII*. This leads to more accumulation of the signal molecule on

the one hand, and to a regulation of many virulence factors through the effector molecule *RNAIII* on the other hand (16) (Fig. 3). In addition to the autoinducing protein AIP, *RNAII* is also induced by RAP (*RNAIII*-activating protein) (5). RAP activates phosphorylation of a 21-kDA protein, which is called TRAP (target of RAP). AIP and TRAP were suggested to activate *RNAIII* synthesis via distinct signal transduction pathways (5). Another protein called SvrA was first postulated to be required in the TRAP pathway (47). However, more recently, Chen and Novick (21) demonstrated that SvrA was not involved in *agr* attenuation but played a role in *S. aureus* infections via an *agr*-independent way.

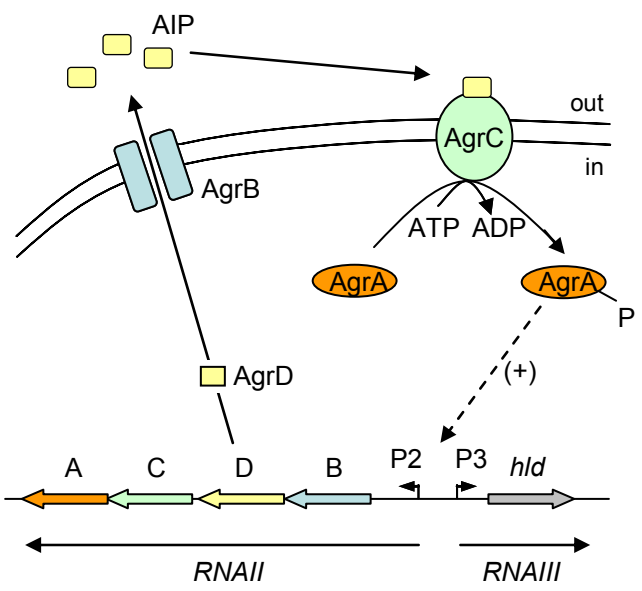

Figure 3: Quorum sensing over the *agr*-system. In an early growth phase, the transcription of *RNAII*, which encodes AgrACDB, is stimulated. The signal molecule AgrD is secreted and processed by the transporter AgrB, yielding the signal molecule AIP (auto inducing protein). When AIP accumulates in the environment, it binds more frequently to the receptor protein AgrC, which then phosphorylates the signal transducer AgrA. The activated AgrA binds to the promoters P2 and P3 and stimulates transcription of *RNAII* and *RNAIII*. This leads to more accumulation of AIP and to regulation of many genes through the effector molecule *RNAIII* (adapted from (16)).

Another important two-component system is the *S. aureus* exoprotein expression locus (*sae*). It is constituted of two co-transcribed genes, *saeR* coding for the response regulator and *saeS,* coding for the sensor protein. It was shown to regulate among others the transcription of *hla, hlb* and *coa* (48). *Coa* codes for coagulase, which belongs to the MSCRAMM family and converts fibrinogen to fibrin.

The staphylococcal respiratory response (*ssrAB* or *srhSR*) locus regulates gene expression in response to oxygen conditions (131). This is important, because *S. aureus* as a facultative anaerobe organism has different growth characteristics depending on the respective oxygen pressure, and some virulence factors need oxygen for their expression (98). The locus codes for the response regulator protein SrrA (SrhS) and the histidine kinase SrrB (SrhR). The system regulates proteins of the energy metabolism but also virulence factors such as TSST.

VraSR mediates response to inhibitors of early and late steps in cell wall peptidoglycan biosynthesis. The VraRS system coordinates the expression of more than 40 genes in *S. aureus* in response to exposure to inhibitory concentrations of cell wall-active

antibiotics, such as glycopeptides and β-lactams. This group of genes is also called the cell wall stress stimulon (8).

The autolysis-related locus regulator/sensor protein ArlRS is a repressor of biofilm formation and influences autolysis. It is also involved in the regulation of *spa* and *hla,* which is probably a result of the regulation of the *sarA* locus by ArlRS (44, 45). ArlRS may also regulate *sarR*, and the σ^B operon and it down-regulates *RNAII* transcription, which in turn leads to a decreased *RNAIII* transcription resulting in downregulation of several virulence genes.

The two-component-system LytRS is involved in autolysis, by positively regulating the expression of *lrgA* and *lrgB*, which are located directly down-stream of *lytS* and *lytR*. LrgA and LrgB modulate the extracellular activity of murein hydrolases and also tolerance to penicillin (18, 51).

WalKR (also YycFG) is an essential two-component system. When down-regulated, cell permeability and susceptibility against macrolide-lincosamide-streptogramin B (MLS_B) antibiotics is increased (83). WalKR positively controls activity of the two major *S. aureus* autolysins, AtlA and LytM, leading to higher autolytic activity (35). WalKR was also shown to directly bind to the promoters of *isaA* and *ssaA*, which encode the immunodominant staphylococcal antigen IsaA, a putative autolysin, and the secreted antigen SsaA, respectively (36). Decreased peptidoglycan biosynthesis and turnover was found when WalKR levels were lowered. WalKR positively controls biofilm formation and was found to play a central role in cell wall metabolism (35).

The staphylococcal accessory regulator SarA and its homologues

The SarA protein is a DNA-binding protein, which was initially described as an activator of the *agr* operon, increasing the levels of *RNAII* and *RNAIII* and thereby altering the expression of virulence factors (22). Later on, it was shown to alter the expression of several cell wall-associated proteins and exoproteins by directly binding to their promoter regions (37).

The *sarA* operon consists of three overlapping transcripts (*sarP1, sarP3* and *sarP2*), which have a common 3' end and are controlled by three different promoters (6). Transcription of *sarP1* and *sarP2* is initiated by the σ^A-specific promoters P1 and P2, while the P3 promoter is dependent on the stress response sigma factor σ^B. All three transcripts encode SarA. Because the expression is dependent on different sigma-factors, expression of *sarA* is allowed during all growth phases: *sarP1* and *sarP2* from early- to mid-exponential growth phase and *sarP3* in the late-exponential and stationary growth-phase or during stress (82).

There are several *sarA* homologues in the *S. aureus* genome. They posses a high level of similarity with SarA and are all regulatory DNA-binding proteins (46). These homologues

include SarR, which regulates *sarA* expression (82), SarH1 (SarS), which binds to the promoter regions of and regulates *hld, ssp, hla* and *spa* (23, 129) and SarT and SarU, which interact with each other and also with *agr* (118). The repressor of toxin, Rot, was shown to upregulate 86 and downregulate 60 genes (116). Other homologues identified are SarY, SarV and SarX (16), but they have not been well characterized, yet.

Alternative sigma factors

The association of the sigma factor with the RNA polymerase (RNAP), forming the holoenzyme, leads to transcription initiation. The sigma factor recognizes specific promoter regions. σ^A is required for the expression of housekeeping genes, while σ^B is involved in the regulation of the gene expression under stress conditions such as starvation and heat shock. σ^B activity itself is regulated by *rsbU, -V* and *–W* gene products. It regulates 251 genes in *S. aureus* (12). Among the regulated genes are *sarA* and *agr.* σ^B increases *sar* expression while simultaneously reducing the *RNAIII* level in a growth phase-dependent manner (13). Another alternative sigma-factor identified in *S. aureus* is a homologue of the *B. subtilis* σ^H*,* which is involved in genetic competence in the latter bacterium (93). In *S. aureus*, an over-expression of σ^H induced the transcription of putative genetic competence genes in two operons, i.e. *comG* and *comE* operons (93).

1.1.4 Biofilms

A biofilm is a sessile community of bacteria that is attached to an artificial or natural surface (34). The capacity of *S. aureus* to form a biofilm is an important virulence factor because it can lead to device-related infections. Embedment in a polymeric matrix protects bacteria from host defences (8), and the altered gene expression of the sessile form renders them refractory to antibiotic treatment (110). The majority of biofilm-mediated device-related infections are caused by either *S. epidermidis* or *S. aureus* (97). Implants such as catheters or heart valves, bear the risk of being colonized with hospital-acquired, multi-resistant organisms. In addition, patients that need foreign devices are mostly compromised by disease or trauma.

Biofilms are formed in multiple steps: attachment of the cells to a surface, also called primary adherence, intercellular accumulation, glycocalyx formation, maturation of the biofilm and escape of the bacteria from the biofilm (50) (Fig. 4).

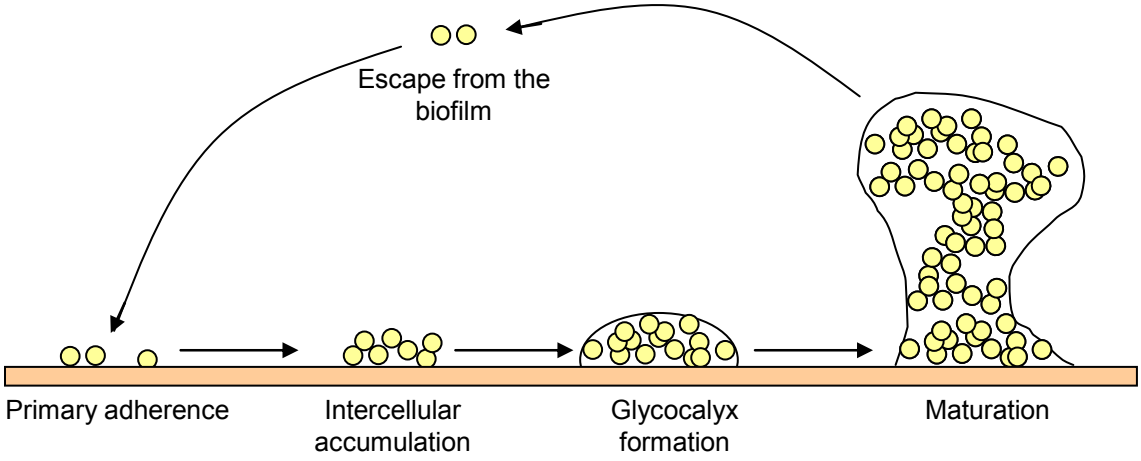

Figure 4: Model of biofilm development. Individual planktonic cells adhere to a surface and can form cell-to-cell-contacts. Slime is produced and the biofilm further maturates. Cells in the biofilm can return to a planktonic lifestyle and complete the cycle of biofilm development (adapted from (100)).

The genetic and molecular basis of biofilm formation is very complex and depends on many factors (reviewed in (96)). Proteins which were shown to be involved in primary adherence include the autolysin Atl (54), the biofilm associated protein Bap (74), the surface protein SasG (26), and other surface-associated adhesins called MSCRAMMS (microbial surface components recognizing adhesive matrix molecules) (43). Teichoic acids have been associated with primary adherence but are at least in *S. epidermidis* also components of the biofilm glycocalyx (52, 115). Another important biofilm component is the so-called polysaccharide intercellular adhesin (PIA), synthesized by proteins encoded by the *icaADBC* operon. PIA was first identified in *S. epidermidis*, but seems to play an important role in *S. aureus* biofilm formation, as well (28). More recently, extracellular DNA (eDNA) has been described as another component of staphylococcal biofilms (109, 111).

Biofilm formation is strongly affected by environmental factors (reviewed in (50, 80, 96)). For example, the amounts of extracellular PIA and teichoic acids depend on growth conditions such as the medium used or on agitation (115). Anaerobiosis stimulates *ica* transcription in *S. aureus* (29); and growth of *S. aureus* during infection of a host results in higher PIA production than under *in vitro* conditions (86). Also the presence of sugars and salts seems to play an important role in the stimulation of biofilm formation (40, 71).

Some global regulators have been shown to be involved in biofilm formation by *S. aureus*. An example is SarA, which was shown to be essential for biofilm formation in several *S. aureus* strains (7). Mutation of the accessory gene regulator (*agr*) was found to affect biofilm formation in some but not all *S. aureus* strains analyzed (99). Although the alternative stress sigma factor σ^B is important in biofilm formation in *S. epidermidis*, it only seems to play a minor role in *S. aureus* biofilm formation (71, 139). WalKR was shown to positively control autolytic activity, in particular that of the two major *S. aureus* autolysins, AtlA and LytM. Concordantly, it was demonstrated to positively control biofilm formation (35). Rbf, a member

of the AraC/XylS family was suggested to be involved in the regulation of the multicellular aggregation step in response to glucose or salt (76). In a wide variety of bacteria, the ubiquitous second messenger cyclic-di-GMP is a regulator of biofilm formation and maintenance (62). Interestingly, amongst a series of established and novel factors found to have an impact on the biofilm forming capacity of *S. aureus*, inactivation of a putative protein with a GGDEF motif of diguanylate cyclases abolished biofilm formation in *S. aureus,* suggesting that cyclic-di-GMP may be involved staphylococcal biofilm formation as well (134).

The understanding of the mechanisms which control biofilm formation in *S. aureus* is crucial to combat this pathogen. Treatments that inhibit the transcription of biofilm controlling genes might be a successful strategy in inhibiting device-related infections in the future.

1.2 Carbon catabolite repression

A global regulatory phenomenon common in virtually all bacteria is carbon catabolite repression (CCR). In CCR, a carbon source in the medium represses the expression of certain genes and operons, whose gene products are needed for the utilization of alternative carbon sources (17). In parallel, genes encoding the required catabolic enzymes are usually expressed only if the respective substrate is present. This mechanism, which is also referred to as carbon catabolite activation (CCA) together with CCR enables the bacterium to use the most energetic carbon source available and thus guarantees the best growth rates. Both regulatory mechanisms are controlled at the level of enzyme activity and transcription of the catabolic genes/operons (128). In most documented cases of CCR, the preferred carbon source is glucose. One well studied exception is the lactic acid bacterium *Streptococcus thermophilus*, which prefers lactose over glucose as the primary carbon and energy source, presenting an adaptation to a special ecological niche (140).

The mechanisms leading to catabolite repression may be quite diverse and have been extensively explored in enteric Gram-negative and low-GC Gram-positive bacteria. The presence of a repressing carbon source can result in lower concentrations of inducers specific for alternate routes of catabolism, in altered activities of specific regulators, or in the activation of global control proteins, such as the catabolite gene activator protein (CAP) in enteric bacteria or the catabolite control protein A (CcpA) in low-GC Gram-positive bacteria. Of these mechanisms, global regulatory circuits mediated by CAP or CcpA were in the main focus of interest, implicating their outstanding importance. However, many aspects of CCR remain to be uncovered, especially in less analyzed, but medically important bacteria, such as *S. aureus.*

1.2.1 The phosphoenolpyruvate-dependent phosphotransferase system

An important role in CCR and CCA plays the phosphoenolpyruvate(PEP)-dependent phosphotransferase system (PTS), which is the main carbohydrate uptake system in low-GC Gram-positive bacteria. The importance is indicated by the presence of fifteen sugar-specific PTS permeases in *B. subtilis* (142). According to the KEGG database (65), *S. aureus* possesses 13 such permeases. In addition to glucose, these permeases specifically transport sucrose, trehalose, lactose, galacitol, L-ascorbate, fructose, mannitol, mannose, maltose, arbutin, *N*-acetylglucosamine and β-glucoside.

The PTS for glucose consists of the enzyme EI, a multidomain complex EIIABC, and the phosphocarrier protein HPr (Fig. 5). Especially important for sensing the physiological state of the cell and the regulation of its nutritional situation is HPr. It can be phosphorylated at two sites, histidine-15 and serine-46. The balance between HPr-His-P and HPr-Ser-P is adjusted as follows: PEP-dependent phosphorylation of HPr by enzyme EI yields HPr-His-P and pyruvate. The phosphoryl group of HPr-His-P is subsequently transferred to enzyme EIIA, and then to EIIB, both soluble domains of the multidomain complex EII. The glucose molecule is transported into the cell by the integral membrane transporter domain and at the same time phosphorylated by EIIB, yielding glucose-6-phosphate. When PTS substrates are metabolized, the level of glycolytic intermediates such as fructose-1,6-bisphosphate rises and stimulates HPr kinase, by the expense of an ATP or a PP_i to generate HPr-Ser-P, the form required for CCR and CCA. Thereupon the rate of sugar import by the PTS is reduced, because HPr-Ser-P is not a substrate for EI and does not participate in the uptake of carbon sources. As soon as the level of glycolytic intermediates drops and the level of inorganic phosphate rises, the bifunctional HPr kinase catalyses the reverse reaction, i.e. dephosphorylation of HPr-Ser-P yielding HPr, which then leads to increased PTS dependent transport again. The phosphorylation state of HPr is thus determined by enzyme EI and HPr kinase. While the activity of EI responds to alterations of the PTS-phosphotransfer activity and the PEP-to-pyruvate ratio in the cell, the activity of the HPr depends on ATP, PP_i, P_i and fructose-1,6-bisphosphate concentrations (32, 132).

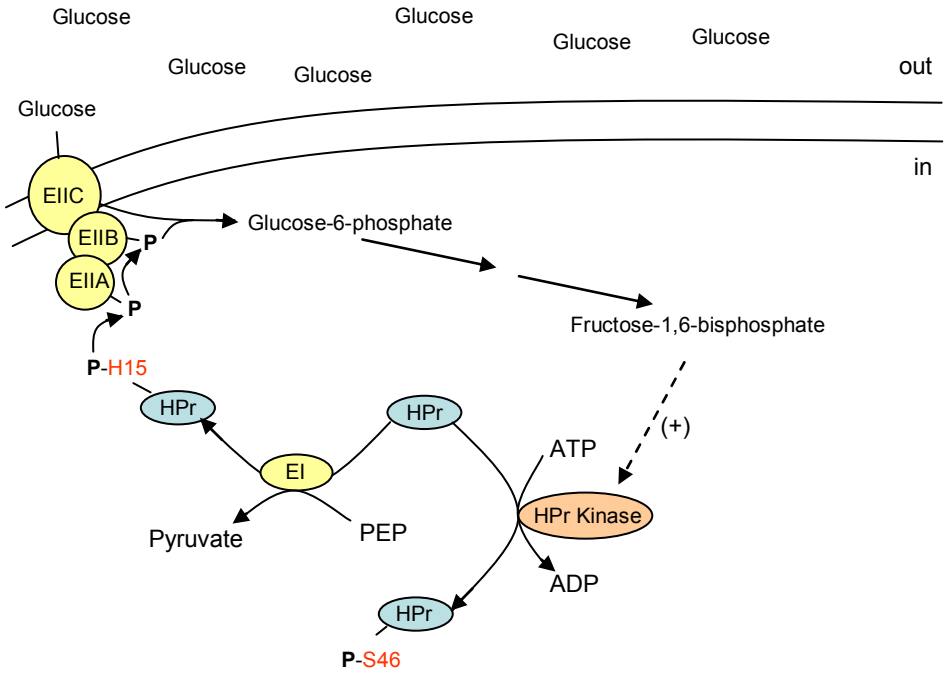

Figure 5: PTS-mediated glucose uptake in the model organism *B. subtilis*. Enzyme EI phosphorylates the phospho-carrier protein HPr at its catalytic histidine residue at the expense of PEP. The phosphoryl group is then transferred to the multidomain complex EIIABC. The glucose molecule is transported into the cell and at the same time phosphorylated, yielding glucose-6-phosphate in the cell. Fructose-1,6-bisphosphate produced from glucose-6-phosphate activates HPr kinase, which then phosphorylates HPr at its regulatory serine residue at the expense of ATP or PP_i (adapted from (142)).

1.2.2 The catabolite control protein A (CcpA)

The catabolite control protein A (CcpA) is a member of the LacI/GalR family of transcriptional regulators mediating CCR in low-GC Gram-positive bacteria (142). It functions as a catabolite repressor or activator. Most knowledge about CcpA comes from investigations of the model organism *B. subtilis* (79, 92, 120, 147). However, CcpA has also been studied in other bacteria such as *Streptococcus mutans* (1), group A *Streptococcus* (3, 123), *Streptococcus thermophilus* (140), *Streptococcus pneumoniae* (57), *Lactococcus lactis* (78, 150), *Lactobacillus casei* (91), and *Staphylococcus xylosus* (59-61).

Gene regulation by CcpA

In *B. subtilis*, activation of CcpA is achieved by interaction with HPr (reviewed in (127)), and this activation is thus interrelated to glucose uptake by the PTS. When the amount of fructose-1,6-bisphosphate is increased in the cell, the phosophorylation of HPr to HPr-Ser-P by HPr kinase is stimulated. HPr-Ser-P then forms a complex with CcpA and increases its affinity for particular *cis*-acting DNA sequences, the so-called *cre*-sites (catabolite-responsive element) (Fig. 6). Whether the transcription of a gene is repressed or activated depends on the location of the *cre* relative to the transcriptional start site. When the *cre* is located

upstream of the -35 region, activation occurs (84, 135), location within the promoter or within the protein coding region might on the other hand lead to prevention of transcription initiation (49) or blockage of the elongation (89, 149). Several general *cre* consensus sequences were proposed for *B. subtilis* (84, 88, 89, 143, 148). The partially degenerated consensus was reported to consist of 14 to 18 base pairs and was similar in all publications. The most recent consensus proposed was WTGAAARCGYTTWWN (W, A or T; R, G or A; Y, T or C; N, A, T, G or C) (88).

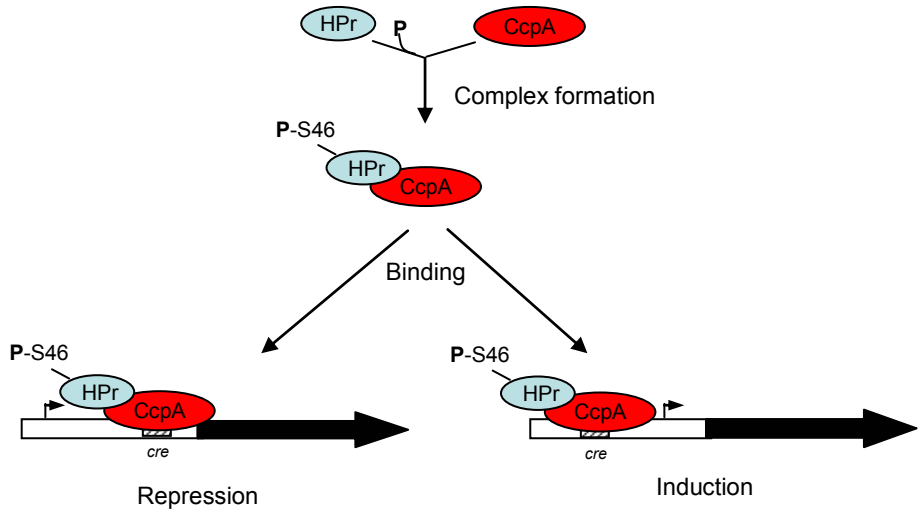

Figure 6: Model of catabolite repression and activation by CcpA in the model organism *B. subtilis*. In the presence of glucose, HPr is phosphorylated at its serine-46 residue and forms a complex with CcpA. The complex then binds to the *cre* (carbon responsive element) and thereby inhibits or induces transcription, depending on the location of the *cre* (adapted from (142)).

In *B. subtilis*, CcpA can not only be activated by HPr-Ser-P, but also by a second mechanism. A closely related protein of HPr, called Crh, can also be phosphorylated by HPr kinase, thereupon binds to CcpA and stimulates DNA-binding. Crh (for catabolite repression HPr) has 45 % sequence identity to HPr. It contains an active-site serine, but no regulatory histidine. Therefore, it has no functionality in PTS-mediated sugar uptake and only acts in CCR. However, a Crh mutant still showed glucose-dependent regulation in *B. subtilis* and the function of this protein is therefore not fully understood. It has been suggested, that Crh might be involved in the repression of genes by metabolites other than sugars (142). However, although *S. aureus* contains homologues for HPr, and HPr kinase, no homologue for Crh can be found in this pathogen (32).

CcpA can apparently also regulate gene expression in the absence of glucose, and consequently also in the absence of HPr-Ser-P or Crh-Ser-P (69, 70, 92). Specific inhibition of *in vitro* transcription by CcpA was promoted by the presence of NADP(H). This effect was not due to binding of CcpA to the *cre*, and therefore NADP(H) is probably not acting as a corepressor (69). Instead, it was proposed that CcpA directly interacts with and inhibits RNA polymerase, a process which is stimulated by NADP(H), independently of the redox state of

the cell (70). However, in *B. megaterium* the overall NADP(H) concentration was shown to be fairly constant (122). Nothing has been published about the overall NADP(H) concentration in *S. aureus* and it is not clear to which signal the interaction of NADP(H) with CcpA responds.

Genes controlled by CcpA

CcpA regulates 10 % of all *B. subtilis* genes, most of which encode metabolic enzymes (92) (Fig. 7). Glycolytic enzymes and genes of the carbon overflow metabolism are induced by glucose. Carbon overflow comprises the metabolization of glucose only as far as pyruvate and acetyl-CoA and subsequent conversion to by-products of metabolism. These by-products are excreted into the environment and comprise lactate, acetate and acetoin in both, *B. subtilis* and *S. aureus* (72, 124). In this way, the cell can generate ATP without using the cytochrome system and maintain its redox balance in the presence of high glucose concentrations. Genes required for gluconeogenesis or for the complete oxidation of glucose, such as genes of the tricarboxylic acid cycle (TCA) cycle or respiration, are repressed (15). However, the role of CcpA goes far beyond the regulation of carbon metabolism, as it also plays a role as regulator of amino acid metabolism. This is indicated for example by the CcpA-dependent repression of the *roc* and *hut* genes, which are involved in the conversion of amino acids into alternative carbon sources (127). These alternative sources are not needed when glucose is present in high amounts.

Two compounds that are of particular importance to the cell are pyruvate and 2-oxoglutarate. Pyruvate can be directly used for the synthesis of several amino acids. Further, it can be metabolized to lactate or acetoin, or it can be converted to acetyl-CoA. Acetyl-CoA can be either metabolized to acetate, introduced into the TCA cycle, or it can be used for the biosynthesis of fatty acids (127). 2-Oxoglutarate plays a role in several diverse pathways. If the cell obtains high amounts of glucose, glutamate, glutamine and other amino acids have to be generated. 2-Oxoglutarate provides the carbon skeleton for these amino acids. If there is no glucose in the medium, 2-oxoglutarate can be the entry point for several amino acids into central metabolism, which can thus be used as secondary carbon sources. Therefore, 2-oxoglutarate is a central link for carbon and nitrogen metabolism. CcpA was shown to be involved in the regulation of both compounds, underlining its importance in metabolism (127).

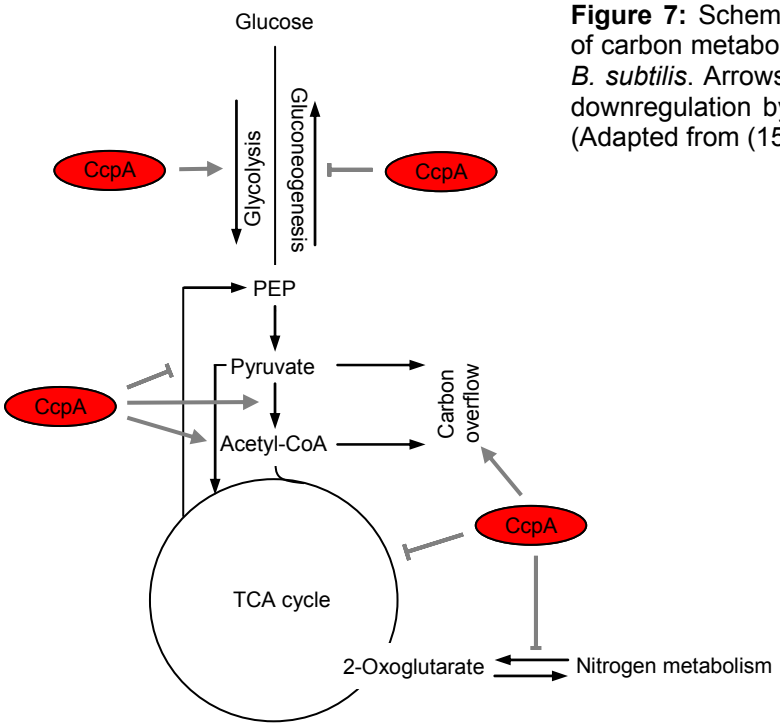

Figure 7: Schematic overview of the main pathways of carbon metabolism subject to regulation by CcpA in *B. subtilis*. Arrows indicate upregulation, bars indicate downregulation by CcpA. PEP, phosphoenolypruvate (Adapted from (15, 127)).

The role of CcpA in pathogenicity and antibiotic resistance

In addition to its role in carbon and nitrogen metabolism, CcpA also seems to regulate several virulence factors and resistance. In group A streptococci, a Δ*ccpA* mutant was significantly less virulent in a mouse model of invasive infection. The mutant was also impaired in colonizing the mouse oropharynx (123). Almengor et *al*. (3) found, that the catabolite control protein CcpA influences expression of the virulence regulator Mga in group A streptococci. Mga is responsible for transcriptional activation of genes involved in adhesion, invasion, and immune evasion. A Δ*ccpA* mutant of *Streptococcus pneumoniae* was attenuated for nasopharyngeal colonization and lung infection in the mouse (57). Abranches et *al.* (1) reported that CcpA had an influence on the expression of traits that are critical for the establishment, persistence, and virulence in *Streptococcus mutans*. CcpA also seems to be essential for biofilm formation by this bacterium (144). In *Streptococcus gordonii*, inactivation of CcpA leads to reduced penicillin tolerance *in vitro* and *in vivo* (14).

Interestingly, De Lencastre et *al*. (31) identified CcpA as influencing methicillin resistance in the *S. aureus* strain COL. This fact together with the observations in those bacteria mentioned above, make CcpA an interesting regulator to analyse in *S. aureus*.

1.2.3 Other regulators of carbon metabolism

In addition to CcpA, several other regulators of carbon metabolism exist in Gram-positive bacteria and many metabolic genes are under the control of more than one regulator. Thus, a complex network is formed, which allows the bacterium to tightly regulate its metabolism in response to minor changes in the environment. Table 2 summarizes regulators of carbon metabolism in *B. subtilis* and shows their homologues in *S. aureus*.

CodY

CodY regulates genes of the nitrogen and carbon metabolism, but also transport and competence genes in *B. subtilis*. It is activated by high levels of GTP and branched chain amino acids (BCAA) in rapidly growing exponential phase cells and thereby represses or activates gene expression. CodY, together with CcpA, is the main regulator of carbon overflow in *B. subtilis* (127). An example for a gene which is upregulated by CodY is *ackA*, which codes for acetate kinase (124). At the same time, the transcription of acetyl-CoA-synthetase (*acsA*) and aconitase of the TCA cycle (*citB*) are repressed by CodY (90). When the level of GTP and BCAAs drops, CodY loses its repressing activity and these genes are derepressed. This leads to the re-introduction of acetate and carbon overflow products and to the activation of the citric acid cycle. *S. aureus* possesses a homologue for CodY, which shares 64 % overall amino acid similarity with the *B. subtilis* CodY. Interestingly, the *S. aureus* CodY was recently shown to negatively regulate virulence gene expression (81).

CcpN

CcpN (YqzB) (for control catabolite protein for gluoneogenic genes) is a control protein of gluconeogenic genes in *B. subtilis* (121). It mediates CCR of *gapB* and *pckA*, two key genes for gluconeogenesis, by directly binding to their promoter regions and repressing their transcription. The presence of a CcpN homologue in *S. aureus* suggests that this CcpA-independent mechanism of CCR is also present in this pathogen.

CggR

The enzymes catalysing the central parts of glycolysis of *B. subtilis*, but also of *S. aureus* are encoded by five genes, *gapA, pgk*, *tpi, pgm*, and *eno*. These genes are co-transcribed together with another gene, called *cggR* in *B. subtilis*, respectively *gapR* in *S. aureus*. This gene, the first cistron of the operon, codes for CggR (for central glycolytic genes regulator), and was found to repress transcription of the *gapA* operon in *B. subtilis*. Fructose-1,6-bisphosphate, which is built in the presence of glucose, inhibits DNA-binding by CggR and thus glycolytic genes are transcribed (33).

CcpB

CcpB, is a regulatory protein of the LacI family, which is involved in the catabolite repression of the gluconate (*gnt*) and xylose (*xyl*) operon in *B. subtilis*. It displays 30 % identity to CcpA, and especially the helix-turn-helix motifs of the two proteins are very similar. Chauvaux et *al*. (19) reported that the activity of CcpB seems to be dependent on the presence of HPr-Ser-P. However, similar sequences to those, which are important for the binding of CcpA to HPr-Ser-P, are absent in CcpB (32), and argue against the assumption made by Chauvaux et *al*.. CcpB was reported to act in parallel with CcpA on catabolite repression but seems to be operative only under highly specific growth conditions (19). No homologue of CcpB was found in *S. aureus*.

CcpC

In *B. subtilis*, a regulator involved in the repression of TCA cycle genes, is CcpC. This LysR-type transcriptional regulator, represses the transcription of the citrate synthase (*citZ)* and aconitase genes (*citB)*. CcpC-activity is induced by citrate and is under the control of CcpA. CcpA-mediated control of CcpC is achieved by the repression of *ccpC* transcription on the one hand and by controlling the amount of citrate on the other hand (67, 68). CcpC also possesses autoregulatory properties. A CcpC-homologue with 34% overall identity can be found in *S. aureus* strain COL, in strain N315 this protein is truncated.

Table 2: Main actors in carbon catabolite repression/activation in *B. subtilis* and *S. aureus*

	B. subtilis[1]	*S. aureus*[2]	Main role
CcpA	BSU29740	SA1557	CCR and CCA of many metabolic genes
HPr	BSU13900	SA0934	Activation of CcpA, part of PTS
HPr Kinase	BSU35000	SA0715	Phosphorylation of HPr and Crh
Crh	BSU34740	No homologue	Activation of CcpA
CcpN	BSU25250 (YqzB)	SA1393	CCR of gluconeogenic genes
CggR	BSU33950	SA0726 (GapR)	CCR of glycolytic genes
CcpB	BSU40870	No homologue	CCR of xylose and gluconate operon
CcpC	BSU14140	SACOL0731 (SA0627*)	CCR of TCA genes
CodY	BSU16170	SA1098	CCR and CCR of many genes of nitrogen and carbon metabolism

[1] according to annotated *B. subtilis* subsp. *subtilis* str. 168 genome, accession nr. NC_000964.
[2] according to annotated *S. aureus* subsp. *aureus* N315 genome, accession nr. NC_002745; COL genome, accession nr. NC_002951.2.
*Truncated.

All these regulatory proteins allow the bacterium to quickly react to changes in the environment. Additional proteins, such as Tnr or RocR, both regulators of nitrogen metabolism, interact with CcpA- and CodY-mediated regulation and add even more complexity to the regulatory network (127).

CcpA-mediated catabolite repression has been demonstrated in *S. xylosus* (59-61). This and the presence of CcpA, HPr and HPr kinase homologues suggest that CcpA acts in a similar way in *S. aureus*. Also homologues for CcpN, CcpC, CodY and CggR (GapR) are present in *S. aureus*. However, no homologues for CcpB, and Crh were found, indicating that CCR and CCA in *S. aureus* might differ from CCR and CCA in the model organism *B. subtilis.*

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2 Aim of this study – Project description

The catabolite control protein A (CcpA) is the main regulator of carbon catabolite repression and activation in low-GC Gram-positive bacteria. It has been extensively studied in the model organism *Bacillus subtilis* but also in other Gram-positives, such as *Streptococcus pneumoniae* or *Staphylococcus xylosus*. In addition to the regulation of metabolic genes, CcpA was found to be involved in the regulation of virulence and antibiotic resistance in some bacteria. Almost nothing is known about the role of CcpA in *Staphylococcus aureus*. An enhanced knowledge of the metabolism of this pathogen on the one hand and a possible connection to virulence on the other hand could help us to better understand and fight this important pathogen. To gain more knowledge about CcpA in *S. aureus*, the following projects were carried out:

Project 1 – *Staphylococcus aureus* CcpA affects virulence determinant production and antibiotic resistance

Antimicrobial Agents and Chemotherapy 2006; 50(4): 1183-1194

CcpA was previously identified as being essential for methicillin resistance in the *S. aureus* strain COL. The aim of this study was to explore the role of CcpA in methicillin resistance in more detail, and to investigate its potential role in virulence. Investigations were carried out to compare the properties of a methicillin resistant *S. aureus* strain with those of its isogenic Δ*ccpA* mutant. The main aspects considered were oxacillin resistance, the expression of several virulence factors, such as alpha-hemolysin (*hla*), surface protein A (*spa*), and *RNAIII*, the effector of the *agr*-locus. The impact of CcpA on capsule formation and glycopeptide resistance were also analysed in different genetic backgrounds.

Project 2 – *Staphylococcus aureus* CcpA affects biofilm formation

Infection and Immunity 2008; 76(5): 2044-2050

S. aureus and *S. epidermids* are both able to adhere to biotic and abiotic surfaces, where they are enveloped in an amorphous slimy material. This ability to form biofilm makes *S. aureus* and *S. epidermidis* among the most frequently isolated pathogens in device-associated infections. Staphylococcal biofilms have been extensively studied and interestingly many of the biofilm assays used in these investigations included high concentrations of glucose. As CcpA controls gene expression in the presence of glucose, the role of CcpA in *S. aureus* biofilm formation was investigated in this study. The capacity to form a biofilm was compared between a *S. aureus* biofilm producer and its isogenic Δ*ccpA* mutant using several methods, such as adherence assays and flow cell experiments. The possible impact of CcpA on several regulators, which are known to affect biofilm formation,

was also analysed. In addition, the impact of the Δ*ccpA* mutation on the formation of different matrix compounds was studied.

Project 3 – *Staphylococcus aureus* CcpA controls *tst* expression

Manuscript in preparation

TSST-1, a superantigen produced by *S. aureus*, is the most common cause of toxic shock syndrome. The regulation of the *tst* gene, which encodes TSST-1, is very complex and is known to be influenced by several environmental factors. As one of these factors is glucose, and as CcpA is known to mediate gene regulation in the presence of glucose, this study focussed on the role of CcpA in *tst* expression. Investigations of *tst* transcription were carried out using promoter reporter gene fusion experiments and Northern blot analyses in different genetic backgrounds, including a Δ*ccpA* mutant. In addition to the transcriptional analyses, the effect of the *ccpA* inactivation was also assessed on the protein level.

Project 4 – Analysis of the CcpA-regulon in *Staphylococcus aureus*

Manuscript in preparation

Several studies have investigated the CcpA transcriptome in different bacteria. These studies, which were mainly carried out in the model organism *B. subtilis,* have underlined the importance of CcpA in the regulation of the central metabolism. In this study, the CcpA transcriptome of *S. aureus* was analyzed for the first time. The investigation mainly focussed on the role of CcpA in metabolism, but also on virulence gene expression in *S. aureus*. These results were compared to those from other Gram-positive bacteria.

3 Results

3.1 Project 1

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Staphylococcus aureus CcpA Affects Virulence Determinant Production and Antibiotic Resistance

Kati Seidl,[1]† Martin Stucki,[1]† Martin Ruegg,[1] Christiane Goerke,[2] Christiane Wolz,[2] Llinos Harris,[3] Brigitte Berger-Bächi,[1] and Markus Bischoff[1]*

Department of Medical Microbiology, University of Zürich, Zürich, Switzerland[1]; Institute for Medical Microbiology and Hygiene, University Hospital Tübingen, Tübingen, Germany[2]; and AO Research Institute, Davos, Switzerland[3]

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Carbon catabolite protein A (CcpA) is known to function as a major regulator of gene expression in different gram-positive organisms. Deletion of the *ccpA* homologue (*saCOL1786*) in *Staphylococcus aureus* was found to affect growth, glucose metabolization, and transcription of selected virulence determinants. In liquid culture, deletion of CcpA decreased the growth rate and yield; however, the effect was only transient during the exponential-growth phase as long as glucose was present in the medium. Depletion of glucose and production of lactate was delayed, while the level of excretion of acetate was less affected and was even higher in the mutant culture. On solid medium, in contrast, growth of the Δ*ccpA* mutant resulted in smaller colonies containing a lower number of CFU per colony. Deletion of CcpA had an effect on the expression of important virulence factors of *S. aureus* by down-regulating *RNAIII*, the effector molecule of the *agr* locus, and altering the transcription patterns of *hla*, encoding α-hemolysin, and *spa*, encoding protein A. CcpA inactivation markedly reduced the oxacillin resistance levels in the highly methicillin-resistant *S. aureus* strain COLn and the teicoplanin resistance level in a glycopeptide-intermediate-resistant *S. aureus* strain. The presence of CcpA in the capsular polysaccharide serotype 5 (CP5)-producing strain Newman abolished capsule formation and decreased *cap* operon transcription in the presence of glucose. The staphylococcal CcpA thus not only is involved in the regulation of carbon metabolism but seems to function as a modulator of virulence gene expression as well.

Carbon catabolite repression (CCR) in bacteria is a widespread, global regulatory phenomenon that allows modulation of the expression of genes and operons involved in carbon utilization and metabolization in the presence of preferred carbon source(s). In CCR, the presence of a preferred carbon source represses the expression of genes and operons whose products are involved in the metabolism of alternative, less-preferred carbon sources. In low-GC gram-positive bacteria, CCR is achieved via transcriptional control, inducer exclusion, and induction prevention (reviewed in references 55 and 60). In this group of bacteria, a common mechanism for transcriptional control has evolved that is mediated via the proteins phosphotransferase HPr, the bifunctional HPr kinase-phosphatase (HPrK/P), and the pleiotropic regulator CcpA (catabolite control protein A). CCR in *Bacillus subtilis* has been studied extensively and is thought to serve as the prototype of CCR-regulated gene expression in gram-positive organisms (reviewed in reference 52). In *B. subtilis*, regulation of transcription of catabolite-repressive genes is exerted mainly through the binding of CcpA to specific *cis*-acting DNA sequences called catabolite-responsive elements (CREs). The DNA-binding activity of CcpA itself is triggered by HPr or its regulatory paralog Crh, which, in the presence of glucose, are phosphorylated by HPrK/P on regulatory seryl residues, in which state they act as cofactors for CcpA. Depending on the localization of the CRE, CcpA may function either as an activator or as a repressor of gene expression. Whole-transcriptome analyses suggest that 10% of all genes in *B. subtilis* are affected in their regulation by glucose by a factor of more than 3, with repressed genes outnumbering activated genes by three to one (3, 39). The majority (80%) of these genes depend on CcpA for regulation, and a recent study indicated that CcpA required interaction with RNA polymerase to inhibit transcription (31).

Although CCR by the catabolite control protein CcpA has been demonstrated in *Staphylococcus xylosus* (reviewed in reference 26), only a little is known about this element in the closely related, pathogenic *Staphylococcus aureus*. However, indications that glucose affects gene expression in *S. aureus* (12, 24, 27, 44, 45, 49), the identification of a potential CRE in the promoter region of the glucose-repressible *pckA* (49) that is highly homologous to the CRE consensus of *B. subtilis* (38), and the presence of HPr (SaCOL1091), HPrK (SaCOL0825), and CcpA (SaCOL1786) homologues in *S. aureus* suggest that a similar mechanism might be present in this pathogen.

Site-directed inactivation of *ccpA* showed here that the lack of CcpA, although causing only minor effects on growth of *S. aureus*, affected oxacillin and glycopeptide resistance and had a significant impact on the ability of *S. aureus* to express virulence factors such as *RNAIII*, *hla*, and *spa* in either the presence or absence of glucose.

* Corresponding author. Mailing address: Department of Medical Microbiology, University of Zürich, Gloriastr. 32, CH-8006 Zürich, Switzerland. Phone: 41 44 634 26 70. Fax: 41 44 634 49 06. E-mail: Bischoff@immv.unizh.ch.
† K.S. and M.S. contributed equally to the article.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and relevant phenotypes are listed in Table 1. When not otherwise specified, bacteria were grown in Luria-Bertani medium (Difco Laboratories, Detroit, Mich.) buffered using 50 mM HEPES (4-2-hydroxyethyl-1-piperazineethanesulfonic acid [pH 7.5]) and a flask volume/culture volume ratio of 5:1 at 200 rpm and 37°C. Where

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype[a]	Reference or source
Strains		
S. aureus		
COLn	Tc^s strain from COL, homogeneous methicillin-resistant strain, Mc^r	30
Newman	Clinical isolate (ATCC 25904), CP5 producer	13
RN4220	NCTC8325-4 $r^- m^+$ *rsbU*	32
KS30	NM143 Δ*ccpA*::*tet*(L), Tc^r	This study
MST04	RN4220 Δ*ccpA*::*tet*(L), Tc^r	This study
MST14	Newman Δ*ccpA*::*tet*(L), Tc^r	This study
MST23	COLn Δ*ccpA*::*tet*(L), heterogeneous methicillin-resistant strain; Mc^r Tc^r	This study
MST31	COLn pAW17; Mc^r Kan^r	This study
MST35	MST23 pAW17; Mc^r Tc^r Kan^r	This study
MST36	MST23 pMST1; Mc^r Tc^r Kan^r	This study
NM143	Newman GISA derivative, in vitro step-selected mutant with a teicoplanin MIC of 24 μg ml^{-1}	N. McCallum; unpublished data
Escherichia coli		
XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tc^r)]	Stratagene
Plasmids		
pAW17	*E. coli-S. aureus* shuttle plasmid with *oris* pAMα1 and ColE1, Kan^r	46
pEC1	pUC19 derivative containing the 1.45-kb ClaI *erm*(B) fragment of Tn*551*; Ap^r Em^r	4
pMR2	pEC1 with a 2.7-kb PCR fragment covering the *ccpA* flanking regions and the *tet*(L) cassette fully replacing the *ccpA* coding region; Amp^r Em^r Tc^r	This study
pMST1	pAW17 with a 1.7-kb PCR fragment covering *ccpA* and its proposed promoter, Kan^r	This study

[a] Abbreviations: Ap^r, ampicillin resistant; CP5, capsular polysaccharide type 5; Em^r, erythromycin resistant; GISA, glycopeptide intermediate resistant *S. aureus*; Kan^r, kanamycin resistant; Mc^r, methicillin resistant, Tc^r, tetracycline resistant.

indicated, mutant strains were grown on antibiotic-supplemented media containing 100 μg of ampicillin, 10 μg of erythromycin, or 10 μg of tetracycline per ml.

DNA manipulations. DNA sequencing, PCR, and plasmid isolation were performed using standard procedures (1) or according to manufacturers' instructions.

Construction of *S. aureus* Δ*ccpA*. A 2.7-kp fragment containing the *ccpA* gene and its flanking regions was amplified by PCR from chromosomal DNA of *S. aureus* COL by use of primer pair ccpABamHI-F/ccpAEcoRI-R (Table 2), digested, and cloned into the BamHI/EcoRI-digested vector pEC1 (4) to generate plasmid pMR1 (Fig. 1). The plasmid was used in a second step to amplify a 5.8-kb

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]	Location (GenBank accession no. NC_002951) or reference
capA167	AGGGTGACAATCCTCAGTTTATGG	153571–153594
capA501	GACTTTAACTGCTGTACCGTCTGCT	153881–153905
capA710	TTGAACCCAATACAGGCAATCC	154035–154056
ccpA-F	CAGCATCCATTGCACTATGC	1830520–1830539
ccpA-R	TCTCTATGGCCACAGTGTCG	1831235–1831254
ccpABamHI-F	gcggatccTAGAATTGCAACAGGTGACG	1829196–1829215
ccpAEcoRI-R	gcgaattCTGTTGCACTTAGTGATGCG	1831924–1831943
ccpAko-F	AATTTCCTCCTTGTAAACG	1831295–1831313
ccpAko-R	ATGGGTGTTGGAAGAATGCC	1830246–1830265
gyr297	TTAGTGTGGGAAATTGTCGATAAT	20
gyr574	AGTCTTGTGACAATGCGTTTACA	20
gyr864	GTACGATTTAATACCGCCCTCATA	20
hla-F	AGAAAATGGCATGCACAAAAA	50
hla-R	TGTAGCGAAGTCTGGTGAAAA	50
MST12	gagtctagaACCAACTGCGAAAGCAGC	1831994–1832011
MST14	gaggatccGGCATTCTTCCAACACCCA	1830246–1830264
pckA-F	CCATCAACTTCTGGATCTGC	1893626–1893645
pckA-R	GGATGTCAGTAGACACATAC	1893129–1893148
RNAIII-F	GTGATGGAAAATAGTTGATGAG	5
RNAIII-R	GTGAATTTGTTCACTGTGTCG	5
spa-F	TgAATTCGTAAACTAGGTGTAGG	46
spa-R	cggTaCCAGGCTTGTTATTGTCTTCC	46
tetL-F	CCTGTTATAAAAAAAGGATC	17
tetL-R	CCATATTGTTGTATAAGTG	17
T7-capA59	taatacgactcactatagggagAATGGAAAGTACATTAGAA	153404–153422
T7-gyr	taatacgactcactatagggagATTATGGTGCTGGGCAAATACA	20

[a] Lowercase letters represent nucleotide additions.

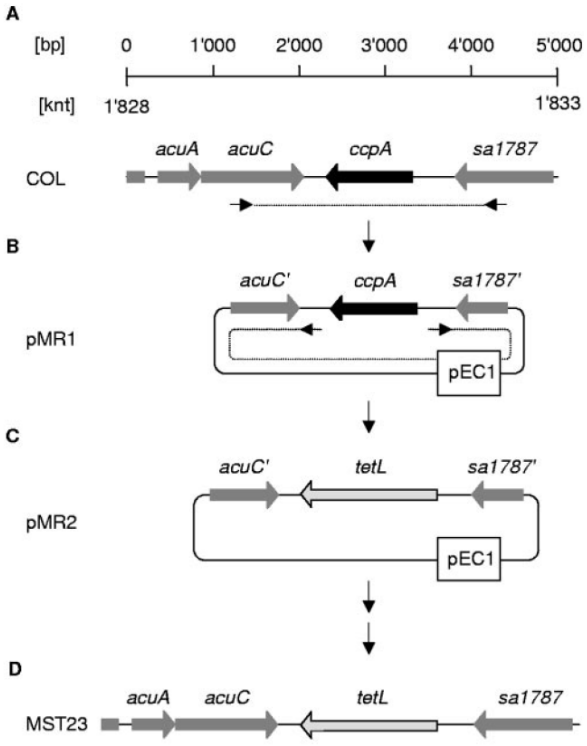


FIG. 1. Schematic representation of the *ccpA* region of *S. aureus* and of the strategy used to obtain MST23 (COLn Δ*ccpA*). The genetic organization of the *S. aureus* COL *ccpA* region (A), pMR1 (B), pMR2 (C), and MST23 (D) is shown. Open reading frame notations and nucleotide numbers correspond to those of the respective genomic regions of strain COL (GenBank accession no. NC_002951). Primers used to amplify the *ccpA* region (A), the *ccpA*-flanking regions including the backbone of pEC1 (B), and the respective PCR products (dotted lines) are indicated.

fragment containing the pEC1 backbone and the regions flanking *ccpA* by use of the primer pair ccpAko-F/ccpAko-R. The 5.8-kb fragment was ligated to a PCR-amplified and phosphorylated 1.7-kb *tet*(L) cassette obtained from plasmid pBT (17) by use of primer pair tetL-F/tetL-R to generate the suicide vector pMR2, and the plasmid was subsequently electroporated into *S. aureus* RN4220. Mutants with the allelic replacement were selected for tetracycline resistance and screened for loss of erythromycin resistance, yielding MST04 (RN4220 *ccpA*::*tet*[L]), which was subsequently used as a donor for transducing the *ccpA* deletion into other *S. aureus* strains.

Construction of plasmid pMST1. A 1.76-kp fragment, covering the *ccpA* gene and 770 bp of its upstream region, was amplified by PCR from chromosomal DNA of *S. aureus* COL by use of primer pair MST12/MST14 (Table 2), digested, and cloned into the BamHI/XbaI site of vector pAW17 (46) to generate plasmid pMST1. The plasmid was first electroporated into *S. aureus* RN4220 and then transduced into the various *ccpA* mutants.

Determination of acetate, glucose, and lactate levels. Aliquots (2 ml) of bacterial cultures were harvested at the indicated time points and centrifuged for 2 min at 16,000 × *g*. The supernatants were incubated at 80°C for 15 min and stored at −20°C until use. Acetate, glucose, and lactate levels were determined with kits from R-Biopharm (Darmstadt, Germany) according to the manufacturer's directions.

Adherence studies. *S. aureus* strains were grown in brain heart infusion (BHI) at 37°C in a shaking water bath at 250 rpm for 3 h to midexponential phase (A_{600} of 1) and used to inoculate 1 ml prewarmed BHI in 24-well plates containing presterilized polyethylene terephthalate (Thermanox) 13-mm disks (Life Technologies, Basel, Switzerland) to a starting A_{600} of 0.05 and incubated without shaking at 37°C for 15 h before fixation for scanning electron microscopy. Fixation and electron microscopy were carried out as described earlier (21).

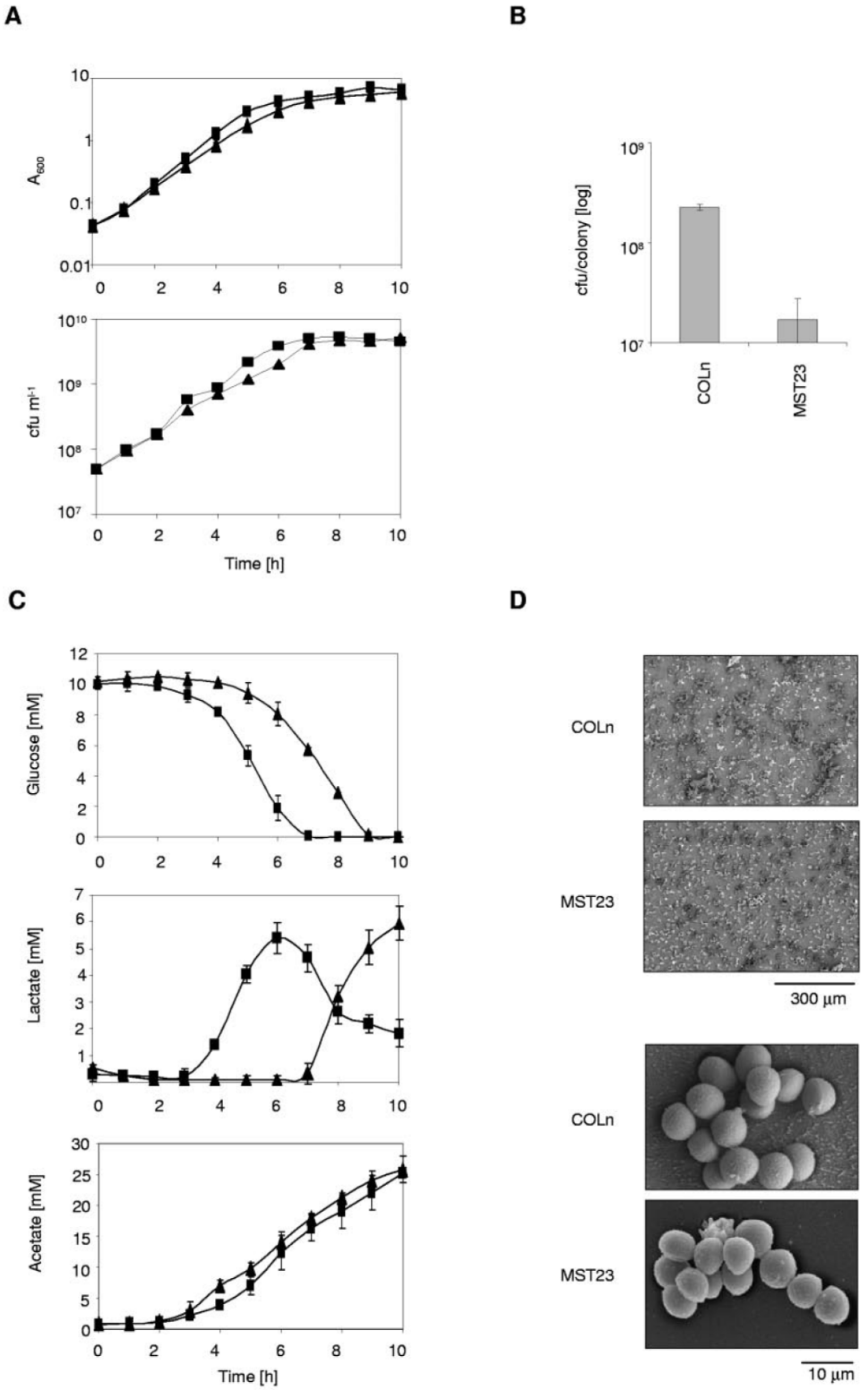
Susceptibility testing. For testing antibiotic resistance on gradient plates, plates were prepared by pouring 35 ml of LB agar containing 1,000 μg ml^{-1} oxacillin into a rectangular inoculum dish (Dynatech, Dübendorf, Switzerland) that was reposed on one side to allow the solidifying LB agar to form a wedge. In a second step, the solidified LB agar plate was placed horizontally and 35 ml of LB agar lacking the antibiotic was poured onto the first layer and allowed to solidify for 3 h, thereby allowing the antibiotic of the lower layer to diffuse into the upper layer to form a gradient. Cells of the strains to be tested were resuspended in physiological NaCl solution to a density of 0.5 McFarland (McF 0.5) and swabbed onto the plate along the gradient. Growth was read after 24 h and 48 h of incubation at 35°C. For E-tests, bacterial suspensions (McF 0.5 for oxacillin and McF 2 for teicoplanin) were swabbed onto the surface of either Mueller-Hinton agar plates supplemented with 2% NaCl (oxacillin) or BHI agar plates (teicoplanin) according to the manufacturer's instructions (AB-Biodisk, Solna, Sweden). Determinations of MICs by broth microdilution were performed as recommended by the CLSI (formerly NCCLS) (9). For population analysis profiles, appropriate dilutions of an overnight culture were plated on LB agar plates containing increasing concentrations of oxacillin (0 to 2,048 μg ml^{-1}) and the numbers of CFU were determined after 48 h of incubation at 35°C.

Northern blot analyses. For in vitro growth studies, overnight cultures of *S. aureus* were diluted 1:100 into fresh prewarmed, HEPES-buffered LB medium (pH 7.5). Cells were grown either with or without 10 mM glucose, samples were removed from the cultures after 1, 3, 5, and 8 h of growth and centrifuged at 13,000 × *g* and 4°C for 5 min, and the cell sediments were snap-frozen in liquid nitrogen. In a second approach, cells were grown in HEPES-buffered LB to an A_{600} of 1, the cultures were split in two, and 10 mM glucose added to one half. Aliquots were sampled at 0, 10, 20, and 30 min and harvested at 16,000 × *g* at room temperature for 1 min, and the cell sediments were snap-frozen in liquid nitrogen. Total RNAs were isolated according to the method of Cheung et al. (6). Blotting, hybridization and labeling were performed as previously described (17). The intensities of the 23S and 16S rRNA bands stained with ethidium bromide were verified to be equivalent in all the samples before transfer. Primer pairs ccpA-F/ccpA-R, hla-F/hla-R, pckA-F/pckA-R, RNAIII-F/RNAIII-R, and spa-F/spa-R (Table 2) were used, respectively, to generate digoxigenin-labeled *ccpA*-, *hla*-, *pckA*-, *RNAIII*-, and *spa*-specific probes by PCR labeling. Data shown were confirmed in at least two independent experiments.

RNA quantification by LightCycler RT-PCR. For quantification of transcripts by LightCycler reverse transcription-PCR (RT-PCR), RNA preparations were performed as described earlier (19). Briefly, approximately 10^9 *S. aureus* cells were lysed in 1 ml TRIzol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) with 0.5 ml of zirconia-silica beads (BioSpec Products, Bartlesville, OK) (0.1 mm diameter) in a high-speed homogenizer (Savant Instruments, Farmingdale, N.Y.). RNA was isolated as described in the instructions provided by the manufacturer of TRIzol. Contaminating DNA was degraded by digesting RNA samples with DNase as described before (19). Sequence-specific RNA standards for quantitative RT-PCR were engineered as described previously (20) using primer pair T7-gyr/gyr864 for *gyrB* and primer pair T7-capA59/capA710 for *capA* (Table 2).

LightCycler RT-PCR was carried out using a LightCycler RNA amplification kit for hybridization probes or with a LightCycler RNA amplification kit and SYBR green I (Roche Biochemicals). Master mixes were prepared following the manufacturer's instructions using the oligonucleotides gyr297/gyr574 for *gyrB* (20) and primer pair capA167/capA501 for *capA* (Table 2). Specific primers were selected in such a way that they bound to an internal part of the respective RNA standard. Standard curves were generated using 10-fold serial dilutions (10^4 to 10^8 copies/μl) of the specific RNA standards. The number of copies of each sample transcript was then determined with the aid of the LightCycler software. At least two independent RT-PCR runs were performed for each sample. The specificity of the PCR was verified by ethidium bromide staining on 3% agarose gels. To check for DNA contamination each sample and RNA standard was subjected to PCR using a LightCycler DNA amplification kit and SYBR green I (Roche Biochemicals). No amplification product was detectable in any of the cases.

Capsular polysaccharide serotype 5 (CP5) detection by indirect immunofluorescence. CP5 production was determined from cultures grown for 24 h in LB medium. Slides with heat-fixed bacteria were washed three times with phosphate-buffered saline (PBS)–0.05% Tween 20 and incubated with 0.2 mg ml^{-1} human immunoglobulin (IgG) (Sigma, Deisenhofen, Germany) diluted in PBS–0.05% Tween 20 for 30 min to prevent unspecific binding of IgG by cell-wall-associated protein A. The slides were incubated with mouse IgM monoclonal antibodies to CP5 (22) diluted 1:50 in PBS–0.05% Tween 20 for 1 h followed by incubation with CY3-conjugated anti-mouse F(ab)$_2$ fragment (Dianova, Hamburg, Ger-


A
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A_{600}
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MST23
Glucose [mM]
Lactate [mM]
Acetate [mM]
Time [h]
COLn
MST23
300 µm
COLn
MST23
10 µm

many) diluted 1:500 in PBS–0.05% Tween 20 for 1 h. Bacteria were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (2 μg ml^{-1}) for 5 min, washed three times with water, and air dried. The slides were then mounted with fluorescent mounting medium (DakoCytomation, Hamburg, Germany), and positively stained bacteria were detected using fluorescence microscopy.

RESULTS AND DISCUSSION

Growth and carbohydrate utilization of *S. aureus* Δ*ccpA* mutants. The *ccpA* gene was deleted in strain RN4220 by allelic replacement (Fig. 1) and transduced therefrom into the capsular polysaccharide serotype 5 (CP5)-producing strain Newman and the methicillin-resistant strain COLn, yielding strains MST14 and MST23, respectively. Analysis of the transcript sizes of the genes surrounding *ccpA*, i.e., *acuAC*, encoding acetoin utilization protein A and C, and *saCOL1787*, thought to encode a chorismate mutase–phospho-2-dehydro-3-deoxyheptonate aldolase, yielded identical patterns for Newman and its Δ*ccpA* mutant MST23, indicating that the genetic manipulations leading to the deletion of *ccpA* did not affect the integrity of the adjacent genes (data not shown).

The growth characteristics and metabolite production of strain COLn and its Δ*ccpA* mutant MST23 were monitored in HEPES-buffered LB supplemented with 10 mM glucose. Comparison of the wild-type and mutant cultures revealed a clear difference only for the mid- to late-exponential-growth phases (i.e., hours 3 to 6). During these growth periods, the mutant displayed slower growth, yielding cell densities lagging approximately 1 h behind the wild-type culture densities. However, the mutant culture reached almost the same A_{600} values after 10 h of growth (Fig. 2A), and no differences in A_{600} values were observable between the wild type and the mutant after 16 and 24 h of growth (data not shown). CFU determinations of the growing cultures showed the same tendency, being different only during the mid- to late-exponential-growth phase, and again, no differences in growth yield were observed after an incubation period of 24 h (data not shown). During the exponential-growth phase, differences in growth rate were also visible with respect to doubling times; the wild type yielded a significantly lower doubling time (44.34 ± 0.65 min) than MST23 (51.67 ± 0.75 min; $P < 0.01$). A clearer difference was observed between the wild type and the mutant on solid media. MST23 produced significantly smaller colonies on sheep blood agar or on Muller-Hinton plates, with lower CFU numbers per colony than COLn after 48 h of incubation (Fig. 2B). No differences were observed when COLn and MST23 were checked for cell size and adherence properties (Fig. 2D), signaling that the differences in growth on solid media were likely to be due to a reduced growth rate of the mutant.

In addition to their growth kinetics, growing COLn and MST23 cultures were further analyzed for glucose metabolism and breakdown. The glucose level of the wild-type culture visibly decreased from hour 3 on and glucose was depleted after hour 7. The glucose level of the MST23 culture started to decrease from hour 5 and was depleted only after 9 h of growth (Fig. 2C). Simultaneously with glucose degradation, the lactate level of the wild-type culture started to increase from hour 3 on, reached its maximum around hour 6, and significantly decreased thereafter. In contrast, the lactate level of the mutant culture started to increase only after 7 h of growth, representing a delay of more than 2 h compared with glucose consumption, and reached its maximum after 10 h. Interestingly, only slight differences were observable between the wild type and the mutant with respect to acetate accumulation; the acetate level seen with the mutant culture preceded that of the wild-type culture by approximately 0.5 h. After 24 h of growth, no lactate was present in either wild-type or mutant media anymore, while acetate levels were reduced to similar amounts, namely, 3.8 ± 0.86 mM for COLn and 3.75 ± 0.76 mM for MST23, signaling that the *ccpA* mutation did not affect the ability of the mutant to assimilate acetate and lactate that was excreted into the media during earlier growth stages.

Although the growth rate and yield of the *ccpA* mutant MST23 were only slightly affected by the deletion, the slower glucose consumption and delayed lactate secretion of MST23, paired with the slightly increased acetate production, suggested that CcpA seemed to exert a positive effect on lactate production and secretion whereas acetate production and secretion seemed to be negatively affected by this protein, taking into account that MST23 possessed lower cell densities and consumed glucose slower than the wild type at almost all time points analyzed. Preliminary Northern analyses supported the hypothesis that CcpA might stimulate lactate and suppress acetate formation in the presence of glucose. Expression of *ldh1* (*saCOL0222*), thought to encode L-lactate dehydrogenase 1 (EC 1.1.1.27) that is believed to catalyze the conversion from pyruvate to lactate when *S. aureus* is grown in the presence of a rapidly catabolizable carbon source under anaerobic conditions, was highly induced in wild-type cells grown in the presence of glucose but was not detectable in the absence of glucose or in the Δ*ccpA* mutant under either growth condition. Expression of genes encoding enzymes involved in acetate formation, such as *pdhABCD*, encoding components of the pyruvate dehydrogenase multienzyme complex thought to catalyze the conversion of pyruvate to acetyl coenzyme A, and the *adhE* homologue (*saCOL0135*) thought to encode alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) that catalyzes the formation of acetyl-coenzyme A to acetaldehyde, on the other hand, was found to be repressed in the wild type in the presence of glucose, while no differences in expression were observed in the Δ*ccpA* mutant in either the presence or absence of glucose (K. Seidl, unpublished data).

Effect of *ccpA* on antibiotic resistance. CcpA was among a series of auxiliary factors reported to reduce methicillin resistance in strain COL upon Tn*551* inactivation (11). We

FIG. 2. Growth characteristics of COLn (squares) and its Δ*ccpA* mutant MST23 (triangles) in LB supplemented with 10 mM glucose. (A) Absorbances at 600 nm (A_{600}) and CFU over the growth cycle. (B) Cfu per colony of COLn and MST23 grown on sheep blood agar for 48 h at 35°C. (C) Glucose, acetate, and lactate concentrations in the culture supernatants corresponding to panel A. The data presented are mean values of three independent experiments. (D) Scanning electron microscopy images of COLn and MST23 adhering to polyethylene terephthalate (Thermanox) disks.

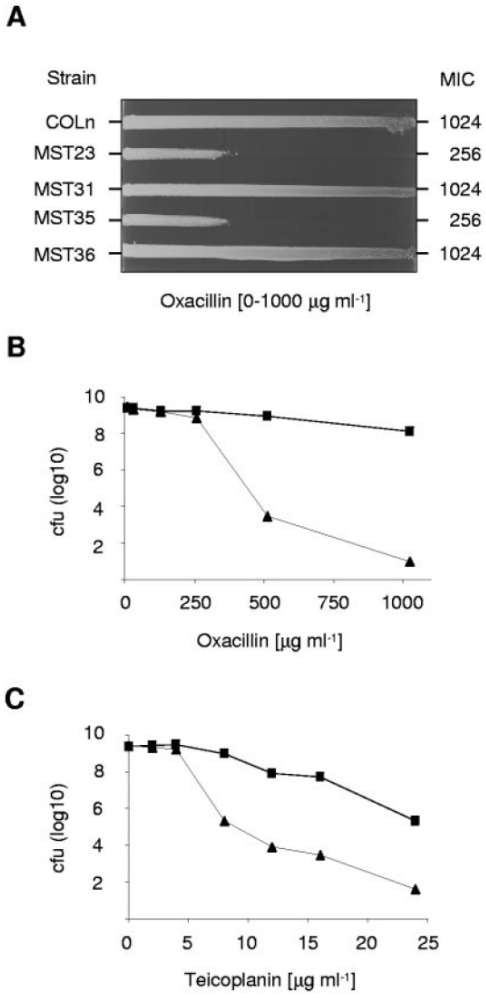

FIG. 3. Susceptibility of COLn and NM143 and their isogenic Δ*ccpA* mutants. (A) Effects of *ccpA* inactivation and complementation on oxacillin resistance. The methicillin-resistant strain COLn, its Δ*ccpA* mutant MST23, control strains MST31 (COLn pAW17) and MST35 [COLn Δ*ccpA*::*tet*(L) complemented with control plasmid pAW17], and strain MST36 [COLn Δ*ccpA*::*tet*(L) complemented with plasmid pMST1] were swabbed along a plate containing an oxacillin gradient. The corresponding MICs of oxacillin (in micrograms per milliliter as determined by E-Test and broth microdilution) for the strains are indicated. (B) Population analysis profiles of COLn (squares) and MST23 [COLn Δ*ccpA*::*tet*(L); triangles] on oxacillin. (C) Population analysis profiles of the step-selected glycopeptide-intermediate-resistant *S. aureus* derivative NM143 (squares) and its Δ*ccpA* mutant KS30 (triangles) on teicoplanin.

demonstrated here a fourfold reduction of the oxacillin MIC for strain COLn upon *ccpA* inactivation and full restoration to its original level by *trans*-complementation with the wild-type *ccpA* allele under the control of its native promoter (Fig. 3A), thus confirming experimentally that the effect was indeed solely CcpA dependent. Moreover, the population analysis profile (Fig. 3B) showed that the homogenous oxacillin resistance of strain COLn was reduced to heterogeneous resistance by *ccpA* inactivation.

Transduction of *ccpA*::*tet*(L) into NM143, the step-selected teicoplanin-resistant derivative of strain Newman, yielding strain KS30, had a similar negative effect on teicoplanin resistance. The *ccpA* inactivation reduced the MIC of teicoplanin from 24 to 12 μg ml^{-1}, and the population analysis profile teicoplanin showed that the number of more highly resistant variants was reduced by a magnitude of over 10^3 (Fig. 3C).

Effect of glucose and *ccpA* on virulence determinant production. Previous studies showed that fermentation of glucose and/or the accompanying decrease in pH affected expression of the global regulator *agr* and of virulence factors such as α-hemolysin (*hla*) and the staphylococcal enterotoxins A, B, and C (*sea*, *seb*, and *sec*) (12, 24, 27, 44, 45). More recently, Weinrick and coworkers (62) showed that mildly acidic conditions (pH 5.5) influenced the expression of a variety of genes, including *agr*, *hla*, and *spa*, encoding protein A, and concluded that changes in staphylococcal gene expression formerly thought to represent a glucose effect might be largely the result of declining pH of the growth medium due to the fermentation of the supplemented carbon source. The effect of glucose on *hla* and *spa* expression is further complicated by the fact that both genes are affected by a complex regulatory network including *agr* and further regulatory elements such as ArlRS (15), MgrA (25), MsrR (46), Rot (37, 47), SaeRS (18, 20), SarA (reference 8 and references therein), SarS (7, 36, 53), SarT (48), SvrA (16), TcaR (36), and the alternative transcription factor σ^B (2, 20, 23), with *rot*, *sae* and *tcaR* expression being pH dependent as well (62).

To elucidate whether *agr*, *hla*, and *spa* transcription was affected by glucose independently from the fermentation-dependent pH and to find out whether CcpA may be involved in mediating such a glucose effect, we monitored the expression levels of these genes in COLn and its *ccpA* mutant MST23, grown in buffered LB in the presence or absence of glucose (Fig. 4). *pckA*, encoding a phosphoenolpyruvate carboxykinase, shown to be affected by glucose, and predicted to be regulated by CcpA in *S. aureus* (49), was included in this study as well. The 50 mM HEPES concentration used here to buffer the medium to pH 7.5 had no inhibiting effect on the growth kinetics and kept the pH fairly constant (Fig. 4A), while concentrations higher than 50 mM were growth inhibitory (data not shown). No changes in pH were observed when COLn and MST23 were grown in LB in the absence of glucose. In the glucose-supplemented wild-type culture, the pH started to drop slightly after 5 h to a final pH of 7, while the pH of the glucose-supplemented MST23 culture dropped from hour 7 on to pH 7.25. Growth of COLn in unbuffered glucose-supplemented LB medium would have caused a drop in pH to 5.5 already after 3 h of growth (data not shown). Addition of glucose produced a higher growth rate and growth yield in the wild type from hour 5 on (Fig. 4A). Surprisingly, glucose seemed to have a slightly inhibiting effect on the growth rate of the mutant MST23 during exponential-growth phase, as seen in the lower A_{600} values for MST23 grown in LB supplemented with glucose compared to those in LB alone; this effect might be due to a slightly increased lag phase.

Expression of *ccpA*, *pckA*, *RNAIII*, *spa*, and *hla* was monitored after 1, 3, 5, and 8 h of growth. In COLn grown in LB,

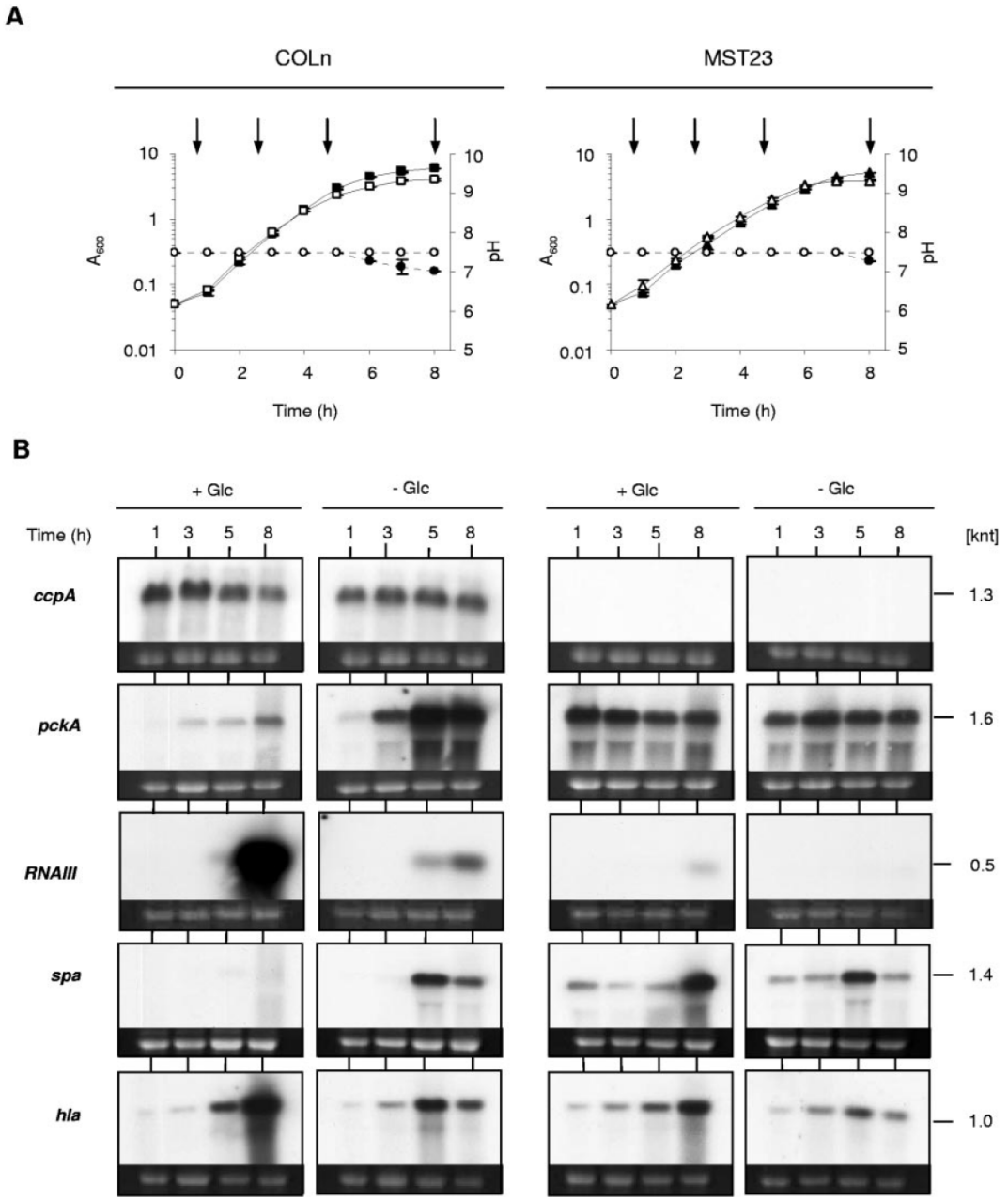

FIG. 4. Northern blot analyses of COLn and its Δ*ccpA* mutant MST23 during growth. (A) Growth characteristics of COLn (squares) and MST23 (triangles) grown in HEPES-buffered LB (open symbols) and HEPES-buffered LB supplemented with 10 mM glucose (closed symbols). At 1-h intervals, an aliquot (2 ml) was removed, the absorbance at 600 nm was measured, and the pH in the culture supernatants (circles) was determined. The results presented are mean values of at least three independent experiments. Time points of sampling for the Northern blot analyses are indicated by arrows. (B) Transcription of *ccpA*, *hla*, *pckA*, *RNAIII*, and *spa* in COLn and MST23 during growth in HEPES-buffered LB (−Glc) and in HEPES-buffered LB supplemented with 10 mM glucose (+Glc). Relevant transcript sizes and time points of sampling are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading.

expression of *ccpA* was found to be fairly constant at all growth stages analyzed (Fig. 4B). Supplementation of glucose seemed to increase the expression of this gene during the early growth stages (i.e., 1 to 3 h), suggesting that expression of *ccpA* might be positively affected by glucose, a phenomenon that has been observed for *ccpA* in other gram-positive organisms (14). No *ccpA* transcripts were detectable in MST23, confirming that the deletion had occurred as intended. Expression of *pckA*

increased with growth and was clearly higher when COLn cells were grown in the absence of glucose, confirming previous findings indicating that *pckA* expression is negatively affected by glucose (49). MST23, on the other hand, already produced *pckA* transcripts at a rather constant and high level early in growth. No significant differences in *pckA* expression were observed in the presence or absence of glucose, signaling that the effect of glucose on *pckA* expression was mediated via CcpA, as has been suggested by Scovill et al. (49). The idea of CcpA-dependent repression of *pckA* transcription is further supported by the presence of a putative CRE in the promoter region of *pckA* (49) that almost perfectly matched (17/18 nt) the CRE consensus of *B. subtilis* (38), suggesting that the CRE sequences might be similar in the two organisms.

Expression of *RNAIII* from the *agr* locus is known to increase during later growth stages (reference 63 and references within). Accordingly, in COLn cells grown in LB, *RNAIII* transcripts were first detected after 5 h of growth and increased with time. Unexpectedly, in glucose-grown cells, a strong increase in *RNAIII* expression occurred at hour 8, contrary to the findings of Regassa et al. (45), who reported unchanged *RNAIII* transcript levels for *S. aureus* cells grown in a fermentor in either the presence or absence of glucose at a constant pH. However, the discrepancy in *RNAIII* expression might be explained by the differing growth conditions, since Regassa and coworkers kept the glucose concentration at a constant level of 100 mM. It is noteworthy that the increase in *RNAIII* expression was detected at a time point when all glucose was exhausted from the medium, indicating that the depletion of glucose might have triggered a signal that induced *RNAIII* transcription, which was not present under the conditions used by Regassa et al. (45). The slight decrease in pH observed in the later growth stages of the wild-type culture in response to glucose (Fig. 4A) could be excluded as a reason for the strong induction of *RNAIII* transcription, since control experiments performed using a fermenter and an equivalent experimental setup allowed the pH to be kept constant and yielded the same induction pattern (data not shown).

No *RNAIII* transcripts were detectable in MST23 cells grown in LB, and addition of glucose yielded only traces of *RNAIII* transcripts after 8 h of growth. The clear differences in *RNAIII* transcript levels observed for COLn and MST23 suggest that the presence of a functional CcpA had a positive effect on *RNAIII* expression. However, this effect was likely to be indirect, since the screening of the *agr* locus did not reveal any apparent CRE in this genomic region that would fit with the CRE consensus of *B. subtilis* (38). Expression of *spa* was noticeably affected by glucose in the wild type. While *spa* transcripts were clearly detectable in COLn during later growth stages (hours 5 to 8) in the absence of glucose, *spa* transcripts were hardly detectable in the presence of glucose (Fig. 4B). However, in MST23, *spa* transcripts were detectable over the whole growth cycle independently of the presence or absence of glucose, although the *spa* transcription profiles seemed to differ between these two conditions to a certain degree. While *spa* expression in MST23 grown in LB peaked around hour 5, analogous to the situation found for the wild type, in the glucose-supplemented LB, *spa* transcription seemed to be highest at the latest time point monitored (i.e., hour 8). Interestingly, significant amounts of *spa* transcripts were already detectable in MST23 during the early growth stages (hours 1 to 3), irrespective of whether glucose was present in the growth media or not, signaling that CcpA might act as a negative regulator for *spa* expression during these growth stages. Expression of *hla* was found to be less affected by glucose and/or CcpA compared with *RNAIII* and *spa* expression results. In COLn grown in unsupplemented LB, *hla* transcripts were detectable from hour 1 on and peaked around the transition from late logarithmic-growth phase to stationary phase. Supplementation of glucose shifted the peak expression of *hla* to the last growth point monitored and seemed to increase the expression level at this growth stage, probably due to the action of *RNAIII*, which was, as shown above, found to be highly expressed under these conditions. Essentially the same *hla* expression patterns as those identified in the wild type were found for MST23, although the overall amounts of *hla* transcripts seemed to be slightly reduced in the *ccpA* mutant.

Since *hla* and *spa* expression are known to be under multiple levels of control, including that by *RNAIII*, it was difficult to judge from the results described above whether the effects observed for *hla* and *spa* were the result of direct CcpA-mediated regulation in response to glucose or whether they might represent a secondary effect of *RNAIII* and other regulatory elements that were not part of this study. To better define the impact of glucose and, in particular, of CcpA on the expression of these two virulence factors, we performed a second series of Northern blot experiments, this time monitoring the expression of *ccpA*, *hla*, *pckA*, and *spa* in cells that were grown to midexponential-growth phase (A_{600} = 1). At this time point, glucose was added to one half of the culture, and cells were harvested in 10-min intervals from 0 to 30 min (Fig. 5). This procedure was likely to abolish the effect of *agr* on *hla* and *spa* expression, since *RNAIII* expression was barely detectable at this time point and under these conditions (data not shown). Moreover, by analyzing the gene expression immediately after the addition of glucose, we assumed that we would be more likely able to identify direct CcpA-dependent effects, since secondary effects were expected to occur with a certain delay. Addition of glucose to the exponentially growing cultures did not result in a temporary growth arrest of these cultures, indicating that the glucose addition did not trigger any inhibition of the primary metabolism (data not shown).

In this second series of Northern blot analyses, the level of expression of *spa* in unsupplemented LB appeared to be roughly constant, only slightly increasing with time, in agreement with the previous findings. Addition of glucose, however, resulted in a clear decrease in *spa* transcription that was already visible after 10 to 20 min in the wild type; this effect was not seen with MST23 (Fig. 5A). Moreover, the levels of *spa* transcripts in MST23 seemed clearly to be higher than those found in COLn, supporting the hypothesis that CcpA of *S. aureus* acts as a direct negative regulator of *spa* expression, either in the absence of glucose or, in a stronger way, in the presence of glucose. Further support for CcpA regulating *spa* expression is given by the fact that the *spa* coding region is preceded by a putative CRE consensus sequence (Fig. 5B). Interestingly, a potential CRE element that perfectly matched with the *B. subtilis* CRE consensus was further identified in the genomic region upstream of the open reading frame of *hla*, signaling that α-hemolysin production might be subjected to a

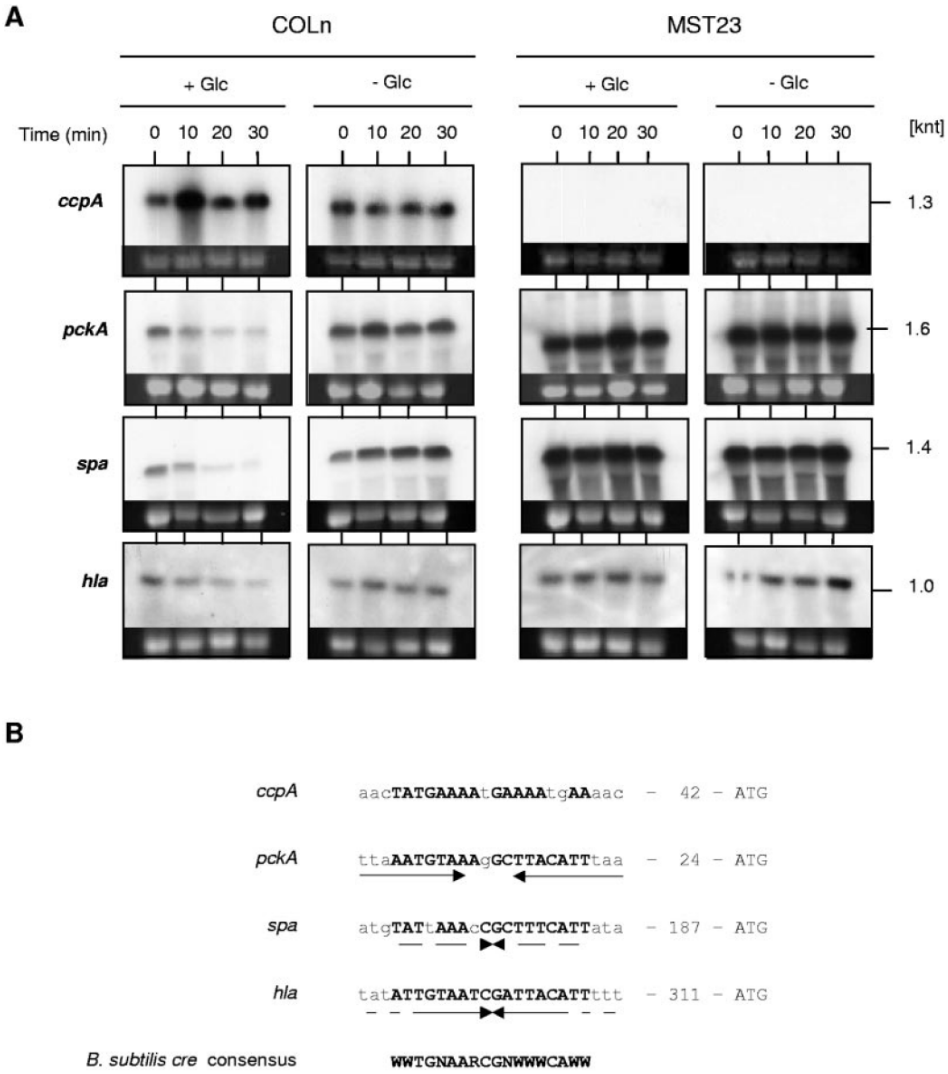

FIG. 5. Effect of glucose addition on the gene expression of COLn and its Δ*ccpA* mutant MST23. (A) Northern blot analyses of *ccpA*, *hla*, *pckA*, and *spa*. Cells were grown in HEPES-buffered LB to midexponential-growth phase (A_{600} = 1), cultures were split in half, and 10 mM glucose was added to one half (+Glc) while the other half was left unchanged (−Glc). Relevant transcript sizes and time points of sampling are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading. (B) Putative CREs identified upstream of *ccpA*, *hla*, *pckA*, and *spa*. Nucleotides fitting with the CRE consensus of *B. subtilis* (38) are highlighted in bold type. Inverted repeats are indicated by arrows. Ambiguity codes are as follows: W denotes A or T, R denotes G or A, and N denotes A, C, G, or T.

CcpA-dependent CCR as well. In line with this assumption, we identified a slight decrease in *hla* transcription in COLn after the addition of glucose that was not detected either in the wild type grown in unsupplemented LB or in the *ccpA* mutant under both sets of conditions (Fig. 5A).

In agreement with the results of the first series of Northern blot analyses, we found *pckA* expression to decrease in the wild type in response to the presence of glucose and to be constant in the absence of this sugar. Equivalent to the situation found with *spa*, expression of *pckA* appeared to be clearly increased in MST23, and addition of glucose again failed to exert an effect on *pckA* expression in the mutant, confirming the findings of Scovill et al. (49) showing that the presence of glucose represses *pckA* transcription and supporting the hypothesis raised by these authors that this negative regulatory effect is exerted via CcpA. Interestingly, expression of *ccpA* itself seems to be positively affected by glucose, since transcription of *ccpA* appeared to be increased in response to the addition of glucose, although this increase was only of a transient nature and was only detectable at 10 min after the sugar was added (Fig. 5A). A potential CRE was identifiable in the promoter region of *ccpA* (Fig. 5B), sharing 15 out of 18 nucleotides with the CRE consensus of *B. subtilis* but lacking the palindromic nature of CREs that was detectable in the CRE candidates of *hla*,

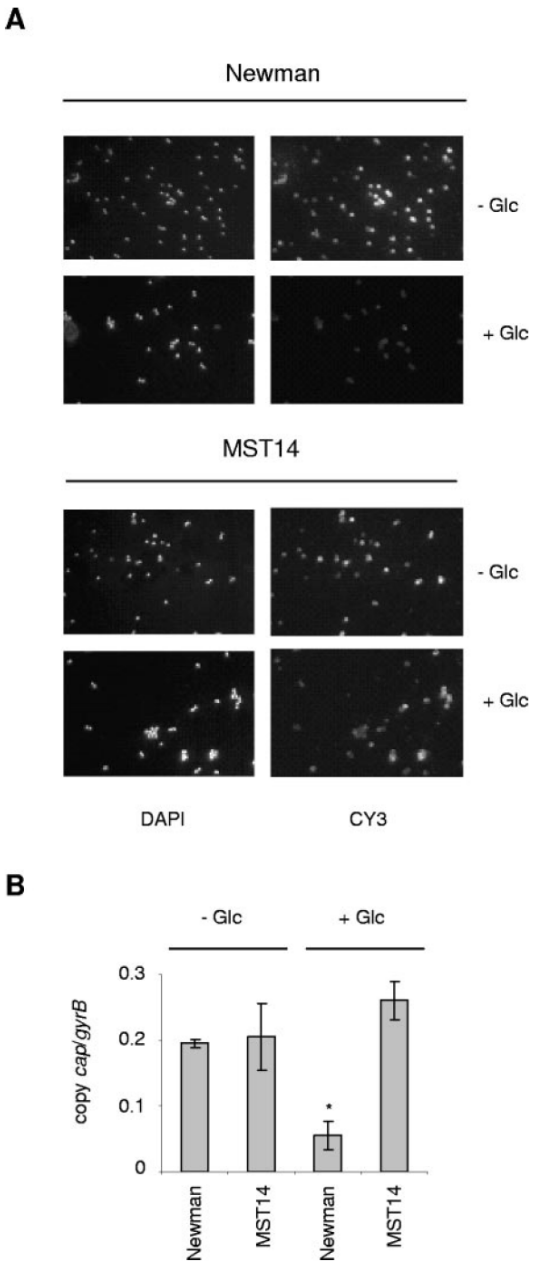

FIG. 6. Capsule production and *cap* expression of Newman and its Δ*ccpA* mutant MST14 in response to glucose. (A) CP5 expression determined by indirect immunofluorescence of strain Newman and its isogenic Δ*ccpA* mutant grown for 24 h at 37°C in HEPES-buffered LB (−Glc), or in HEPES-buffered LB supplemented with 10 mM glucose (+Glc). Bacteria were stained with 4′,6′-diamidino-2-phenylindole (DAPI), and marked with CP5-specific monoclonal antibodies and stained with Cy3-conjugated anti-mouse antibodies (CY3). (B) Quantitative transcript analysis of *capA* by LightCycler RT-PCR of strain Newman and its isogenic Δ*ccpA* mutant grown for 8 h at 37°C in HEPES-buffered LB (−Glc) or in HEPES-buffered LB supplemented with 10 mM glucose (+Glc). Transcripts were quantified in reference to the transcription of gyrase (in copies per copy of *gyrB*). Values from two separate RNA isolations and two independent RT-PCRs each were used to calculate the mean expression (±standard errors of the mean). Glc, glucose; asterisk, $P < 0.05$ for Newman without Glc, MST14 without Glc, and MST14 with Glc.

pckA, and *spa* and leaving the question currently open of whether the observed increase in transcription was mediated via CcpA by itself or not.

Effect of *ccpA* and glucose on capsular polysaccharide production. The majority of clinical isolates produce capsular polysaccharides of serotype 5 (CP5) or serotype 8 (CP8), which protect *S. aureus* against opsonophagocytic killing by polymorphonuclear leukocytes (28, 29, 34, 54, 59) and have been shown in a number of animal models of infection to enhance its virulence (40, 43, 54, 56, 61). Expression of CPs is influenced by various environmental signals in vitro and in vivo (reviewed in references 41 and 59), and transcription of the *cap* operon was shown to be affected by regulatory elements such as *agr*, *mgr*, *sae*, *sarA*, and the alternative σ-factor σ^B (2, 10, 33, 34, 35, 42, 51, 57). Both biosynthetic pathways for CP5 and CP8 production utilize precursors of the cell wall, such as UDP-*N*-acetylglucosamine, suggesting that CP-producing proteins might compete with cell-wall-producing enzymes for the availability of UDP-*N*-acetylglucosamine, thereby affecting the carbon flux of *S. aureus*. We therefore analyzed whether glucose, and specifically *ccpA*, might affect CP synthesis, as has been shown recently for the low G+C-content gram-positive pathogen *Clostridium perfringens* (58).

Since the CP serotype 5 strain COLn was found to produce only a little CP under the conditions tested (C. Wolz, unpublished data), the CP5 prototypic strain Newman and its Δ*ccpA* derivative MST14 were used to investigate the effect of glucose and CcpA on CP formation (Fig. 6). Newman wild-type and MST14 cells were grown for 24 h in LB medium in the presence or absence of 10 mM glucose, and the CP5 production was determined by indirect immunofluorescence using monoclonal antibodies raised against CP5 (Fig. 6A). In the absence of glucose, most of the wild-type cells produced CP5. However, in the presence of glucose, CP5 was abolished, indicating that glucose repressed CP formation. Interestingly, CP5 production in the *ccpA* mutant MST14 was not affected and was present irrespective of the presence or absence of glucose in the growth medium. The immunofluorescence data were confirmed by real-time PCR (Fig. 6B). The expression of the *cap* operon was almost indistinguishable between the wild type and mutant in the absence of glucose, whereas in the presence of glucose, strain Newman produced significantly fewer *cap* transcripts ($P < 0.05$) than MST14, which expressed *cap* in roughly the same amounts as in the absence of glucose. Both findings strongly suggested that the presence of glucose repressed CP formation and that this effect was, at least in part, mediated via CcpA on the transcriptional level, adding a further regulator to the complex network of regulatory elements and environmental conditions that control *cap* operon expression. However, since no apparent CRE was identifiable within the genomic region encoding the *cap* operon, it is again likely that the CcpA effect on *cap* transcription was of an indirect nature and might be mediated by downstream regulators.

Concluding remarks. Deletion of *ccpA* had a clear impact on the expression of *RNAIII* and on virulence factors of *S. aureus*, some of which have previously been shown to be affected by glucose. Interestingly, the deletion of *ccpA* produced an effect on gene expression not only in the presence but also in the absence of glucose, indicating that the function of CcpA might not be restricted to CCR. Our findings that CcpA of *S. aureus* influenced the transcription of at least five genes and operons, with most of them being involved in virulence of this pathogen, suggests that CcpA might represent an important global regulator of gene expression in *S. aureus* that, like that of its homologue in *B. subtilis*, may not be limited to regulating carbon uptake and metabolization. A preliminary computational screening of the *S. aureus* COL genome with the CRE consensus of *B. subtilis* (38) indeed indicated more than 110 CREs to be present in the promoter or N-terminal coding regions of genes and operons encoded by *S. aureus*, if allowing one mismatch to occur. Whole genome and proteomic analyses are currently ongoing to identify the CcpA regulon in *S. aureus*.

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3.2 Project 2

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Staphylococcus aureus CcpA Affects Biofilm Formation▽

Kati Seidl,[1] Christiane Goerke,[2] Christiane Wolz,[2] Dietrich Mack,[3] Brigitte Berger-Bächi,[1] and Markus Bischoff[1,4]*

Institute of Medical Microbiology, University of Zürich, Zürich, Switzerland[1]; Institute for Medical Microbiology and Hygiene, University Hospital—Tübingen, Tübingen, Germany[2]; University of Wales—Swansea, Swansea, United Kingdom[3]; and Institute of Medical Microbiology and Hygiene, University of Saarland Hospital, Homburg/Saar, Germany[4]

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Biofilm formation in *Staphylococcus aureus* under in vitro growth conditions is generally promoted by high concentrations of sugar and/or salts. The addition of glucose to routinely used complex growth media triggered biofilm formation in *S. aureus* strain SA113. Deletion of *ccpA*, coding for the catabolite control protein A (CcpA), which regulates gene expression in response to the carbon source, abolished the capacity of SA113 to form a biofilm under static and flow conditions, while still allowing primary attachment to polystyrene surfaces. This suggested that CcpA mainly affects biofilm accumulation and intercellular aggregation. *trans*-Complementation of the mutant with the wild-type *ccpA* allele fully restored the biofilm formation. The biofilm produced by SA113 was susceptible to sodium metaperiodate, DNase I, and proteinase K treatment, indicating the presence of polysaccharide intercellular adhesin (PIA), protein factors, and extracellular DNA (eDNA). The investigation of several factors which were reported to influence biofilm formation in *S. aureus* (*arlRS*, *mgrA*, *rbf*, *sarA*, *atl*, *ica*, *citZ*, *citB*, and *cidABC*) showed that CcpA up-regulated the transcription of *cidA*, which was recently shown to contribute to eDNA production. Moreover, we showed that CcpA increased *icaA* expression and PIA production, presumably over the down-regulation of the tricarboxylic acid cycle genes *citB* and *citZ*.

Staphylococcus epidermidis and *Staphylococcus aureus* are the most frequent causes of foreign body-associated infections, mainly due to their ability to form an adherent, multilayer bacterial biofilm on all sorts of surfaces. Embedment in a polymeric matrix protects bacteria from host defenses (3), and the altered gene expression of the sessile form (43) renders them refractory to antibiotic treatment.

Biofilm formation is a multistep process, characterized by attachment of the cells to a surface by physicochemical interactions, which is followed by growth-dependent intercellular accumulation, glycocalyx formation, maturation of the biofilm, and finally escape of the bacteria from the biofilm (22). Besides the overall cell charge and hydrophobicity, which can affect initial attachment to various surfaces, staphylococci possess an impressive number of surface-associated adhesins (microbial surface components recognizing adhesive matrix molecules, or MSCRAMMS) to adhere to the host's matrix proteins (20). The genetic and molecular basis of biofilm formation in staphylococci is multifaceted (reviewed in reference 39), and the composition of the polymeric biofilm matrix is complex and varies from strain to strain (9).

An important component of many *S. epidermidis* biofilms is the polysaccharide intercellular adhesin, PIA, also termed polymeric *N*-acetylglucosamine (PNAG), which is synthesized by the *icaADBC*-encoded proteins (34, 35). PIA is also produced by *S. aureus* (13), and the *ica* operon appears to be present in virtually all *S. aureus* strains (17, 29, 46). However, the role and importance of PIA in different clinical settings are not completely understood (46). Biofilm formation can also occur in several PIA-independent ways (2): The teichoic acids, surface-exposed charged polymers, which constitute an important part of the *S. aureus* cell wall, function in primary adherence (23, 57) and are a component of the biofilm matrix as well (49). Proteinaceous factors also contribute significantly to primary attachment and/or promote biofilm formation. Among them are the bifunctional autolysin Atl, which is involved in cell separation (4, 24); possibly also the related cell wall-associated proteins SasG and Pls (12); the biofilm-associated protein Bap, which is mainly found in bovine *S. aureus* lineages (31); and the aggregation-associated protein Aap from *S. epidermidis*, which, upon proteolytic processing, induces a PIA-independent biofilm-positive phenotype (47). Functions in biofilm formation were reported also for α-hemolysin, a secreted toxin, but required for cell-cell interactions and biofilm formation (5), as well as for FmtA (16, 55), a penicillin-binding-like protein that plays a role in methicillin resistance (30). Besides proteins, extracellular DNA (eDNA) seems to be a major structural component in staphylococcal biofilms (26, 42, 44).

The regulation of biofilm formation is complex and is influenced by various regulatory systems. The alternative stress sigma factor σ^B is important in biofilm formation in *S. epidermidis* but plays a minor role in *S. aureus* (29, 56). The staphylococcal accessory regulator SarA, which controls the synthesis of certain virulence factors directly or via the *agr* system and which itself is partly controlled by σ^B, is essential for biofilm formation in several *S. aureus* strains (1). Mutations of the accessory gene regulator (*agr*) were found to affect biofilm formation in some but not all *S. aureus* strains analyzed (40). A

* Corresponding author. Mailing address: Institute of Medical Microbiology and Hygiene, University of Saarland Hospital, 66421 Homburg/Saar, Germany. Phone: 49 6841 162 39 63. Fax: 49 6841 162 39 85. E-mail: markus.bischoff@uniklinikum-saarland.de.
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TABLE 1. *S. aureus* strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype[a]	Source or reference
Strains		
MST04	RN4220 *ccpA*::*tet*(L); Tc^r	50
SA113	ATCC 35556; PIA-dependent biofilm producer	25
KS66	SA113 *ccpA*::*tet*(L); Tc^r	This study
KS66 compl	KS66(pMST1); Kan^r; Tc^r	This study
KS66 empty	KS66(pAW17); Kan^r Tc^r	This study
SA113 Δ*ica*	ATCC 35556 Δ*ica*::*tet*; Tc^r; deletion of the *icaADBC* operon	13
DSM 20231	Cowan serotype 3	52
KS153	DSM 20231 *ccpA*::*tet*(L); Tc^r	This study
KS153 compl	KS153(pMST1); Kan^r Tc^r	This study
Plasmids		
pAW17	*Escherichia coli-S. aureus* shuttle plasmid with ori pAMα1 and ori ColE1; Kan^r	48
pMST1	pAW17 with 1.7-kb PCR fragment covering *ccpA* and its proposed promoter; Kan^r	50

[a] Abbreviations: Tc^r, tetracycline resistant; Kan^r, kanamycin resistant.

TABLE 2. Primers used in this study

Primer	Sequence (5′→3′)	Source or reference
arlRSprobe+	TCGTATCACATACCCAACGC	This study
arRSprobe−	GAGTATGATGGACAAGACGG	This study
citBprobe+	CAGAGGTGTACCAGCCG	This study
citBprobe−	GGTTGTCCAAGCATTCCAG	This study
citZprobe+	CATCTGACAATGATGATACC	This study
citZprobe−	GGAGTATGTTACAGATCACG	This study
rbfprobe+	TGATTTACGTGACGAGCTCG	This study
rbfprobe−	GCACTATTACTTAAATCTCG	This study
atlprobe+	CCAAGGAACCATTGATAAGC	This study
atlprobe−	TGATACTGCTAAACCTACGC	This study
mgrAprobe+	TCTTGAGATAAAGAAGAAGC	This study
mgrAprobe−	GAAGTACAATCTAACATACC	This study
*cidA*1-F	CCCATATGCACAAAGTCCA ATTA	59
*cidA*1-R	CCCTCGAGTTCATAAGCGTCT ACACC	59
Sa*sarA*f	AGGGAGGTTTTAAACATGGC	9a
Sa*sarA*r	CTCGACTCAATAATGATTCG	9a
16S-F	CGGAGTGCTTAATGCGTTAG	This study
16S-R	CAATGGGCGAAAGCCTG	This study

two-component system which positively controls biofilm formation in *S. aureus* is WalK/WalR, also known as YycG/YycF (15), which plays an important role in cell wall modeling through activation of several genes involved in cell wall degradation.

Several environmental factors have been reported to affect biofilm formation (reviewed in references 22, 33, and 39). Anaerobiosis stimulates *ica* transcription in *S. aureus* (14), and growth of *S. aureus* during infection of a host results in higher PIA production than under in vitro conditions (37). The relative amounts of extracellular PIA and teichoic acids depend on growth conditions such as the choice of the medium or on agitation (49). Especially the presence of sugars seems to play an important role in the stimulation of this process (18, 29). The impact of glucose in the induction of biofilm formation in *S. aureus* is also reflected by the fact that most of the biofilm adherence assays used in previous studies included high concentrations of either glucose or sucrose (1, 5, 13, 14, 19, 27, 32, 51, 54). Rbf, a member of the AraC/XylS family, was recently suggested to be involved in the regulation of the multicellular aggregation step of *S. aureus* biofilm formation in response to glucose or salt (32).

We recently showed the impact of the catabolite control protein A (CcpA) on carbon metabolism, up-regulation of certain virulence determinants, and resistance to cell wall-directed antibiotics (50). Since the activity of CcpA is activated in the presence of glucose or sucrose (27), and since CcpA was shown to affect biofilm formation in *Bacillus subtilis* and *Streptococcus mutans* (7, 53, 58), we wondered about its role in biofilm formation in *S. aureus*.

In this study, we deleted the *ccpA* gene in strain SA113, a biofilm former known to produce PIA (13). We show that, depending on the growth medium, SA113 was able to form a strong biofilm and that the deletion of *ccpA* reduced its biofilm formation capacity and PIA production.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *S. aureus* strains, plasmids, and primers used in this study are listed in Tables 1 and 2. All strains generated for this study were confirmed by Southern blot analysis and pulsed-field gel electrophoresis of total genome SmaI digests.

Biofilm assays. Biofilm formation under static conditions was monitored as described in reference 32. Briefly, Trypticase soy broth (TSB), brain heart infusion (BHI), or LB medium (Becton Dickinson), supplemented with different amounts of glucose, was inoculated 1:200 with an overnight culture. Two hundred microliters of this suspension was transferred to wells of 96-well Nunc Delta tissue culture plates (Roskilde, Denmark) and incubated for 18 h at 37°C. After incubation, the wells were rinsed with 200 μl phosphate-buffered saline three times, air dried, stained with 0.1% safranin for 30 s, and washed three times with distilled water. The adhering dye was dissolved with 30% acetic acid, and the absorption was measured at 530 nm in a microtiter dish reader (Powerwave XS; BioTek). Analoguous experiments were carried out in Nunc 12-well plates using 1 ml of the corresponding suspensions or solutions for a better visual observation.

Biofilm formation under flow conditions was determined basically as described by Beenken et al. (1) using TSB supplemented with 1% glucose, uncoated three-channel flow cells (total volume, 160 μl), and a flow rate of 0.5 ml min^{-1} for 24 h.

Growth was monitored in 10-ml glass test tubes containing 2 ml of TSB supplemented with 1% glucose and inoculated 1:200 with preculture. Cells were grown for 18 h at 37°C with shaking (180 rpm).

Biofilm stability against protease, sodium metaperiodate, and DNase I treatment. Biofilm stability assays were carried out in Nunc 12-well plates as described by Toledo-Arana et al. (54), using the growth conditions described above and TSB supplemented with 1% glucose. After 18 h, the medium was removed and substituted with fresh medium supplemented with either 100 μg/ml protease K or 10 mM of sodium metaperiodate and incubated for 2 h at 37°C. Medium without supplement served as a negative control. To test the impact of DNase I, cells were treated as described by Rice et al. (44). Briefly, cells were grown in TSB supplemented with 1% glucose in the presence of 140 U/ml RNase-free DNase I (Fluka, Switzerland) in test tubes or in Nunc plates for 18 h at 37°C. Addition of DNase I had no effect on the growth rates of cells grown in liquid culture.

Growth on CRA. Congo red agar (CRA) screening was performed basically as described by Knobloch et al. (29). Cells grown on blood plates were diluted to McFarland standard of 0.5, stamped on plates made of TSB agar supplemented with 1% glucose and 0.08% Congo red, and incubated for 18 h at 37°C. MICs of Congo red were determined by broth microdilution modified according to CLSI guidelines (11) in TSB supplemented with 1% glucose.

PIA determination. PIA production was monitored by analyzing the cell surface extracts from cultures grown for 2 and 8 h in TSB supplemented with 1% glucose. PIA was extracted as described by Cerca et al. (6). Detection was performed using a rabbit polyclonal anti-PIA antibody (19) after blocking with human immunoglobulin G.

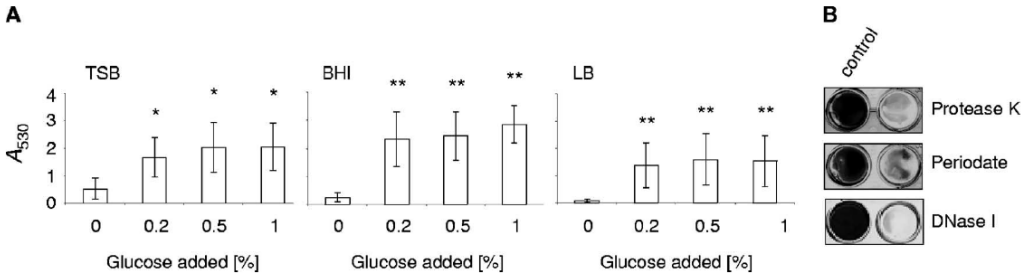

FIG. 1. (A) Quantification (A_{530}) of biofilm formation of strain SA113 in different media in response to glucose. Results represent the averages of at least three independent experiments. Error bars indicate the standard deviation of the mean A_{530}. * and **, $P < 0.05$ and $P < 0.01$, respectively, for unsupplemented versus supplemented cultures. The media contained the following concentrations of glucose and NaCl before glucose addition: TSB, 0.25% glucose and 0.5% NaCl; BHI, 0.2% glucose and 0.5% NaCl; and LB, 0% glucose and 1% NaCl. (B) Biofilm stability assays of SA113. Preformed biofilms were treated for 2 h with either sodium metaperiodate (10 mM) or proteinase K (100 μg/ml). For the DNase I stability assay, SA113 cells were grown for 18 h in the presence or absence of 140 U/ml DNase I.

Primary adherence measurements. Primary adherence was measured by diluting cells in the stationary growth phase in TSB supplemented with 1% glucose to obtain approximately 30 CFU/ml. Two milliliters of the appropriate dilutions was added to Nunc Delta six-well-plates and incubated for 30 min at 37°C. In parallel, cells were plated on blood plates to determine the original number of cells applied to the microtiter plates. The six-well plates were rinsed gently three times with 5 ml of sterile phosphate-buffered saline (pH 7.4) and covered with 3 ml of molten BHI agar (0.8%). Primary attachment was expressed as a percentage of CFU on the six-well-plates compared to the CFU on blood plates.

Quantification of *icaA* transcription. The *icaA* and *gyrB* transcripts were quantified by LightCycler reverse transcription-PCR as described earlier (19) using RNA samples obtained from cultures grown for 2 and 8 h in TSB supplemented with 1% glucose at 37°C and 200 rpm.

Northern blot analyses. For Northern blot analyses, cells were grown in TSB supplemented with 1% glucose and harvested after 2 h of growth, with both both wild type and mutant having reached an optical density at 600 nm of approximately 0.4. Cells were centrifuged for 2 min at 12,000 × *g*, and cell sediments were snap-frozen in liquid nitrogen. RNA isolation and Northern blotting were performed as described earlier (36). The primer pairs used to generate digoxigenin-labeled *arlRS*-, *citB*-, *citZ*-, *rbf*-, *cidA*-, *mgrA*-, *atl*-, and *sarA*-specific probes by PCR labeling are shown in Table 2. All Northern blot analyses were performed at least twice on independently isolated RNA samples. An internal 0.5-kb fragment of the 16S rRNA genes (nucleotides 2232818 to 2233328 of GenBank accession no. CP000046) was used to probe the 16S rRNA gene as a loading control.

Triton X-100-induced autolysis assays. Autolysis assays were performed as described by Fournier and Hooper (21). Bacteria were grown in TSB supplemented with 1% glucose for 2 h. Cells were pelleted by centrifugation and resuspended in 50 mM Tris-HCl (pH 7.5)–0.1% Triton X-100 to obtain an A_{600} of approximately 1. The cells were then incubated at 30°C with shaking, and changes in A_{600} and CFU were monitored. Results were normalized to CFU at time zero (CFU_0): i.e., % living cells at time t = (CFU at time t/CFU_0) × 100.

RESULTS AND DISCUSSION

Effect of glucose on biofilm formation in *S. aureus* SA113. Biofilm formation of strain SA113 in different complex growth media (TSB, BHI, and LB) was shown to be promoted by adding increasing concentrations of glucose. Without glucose supplementation (Fig. 1 A), no significant biofilm formation (A_{530}, <0.5) was observed irrespective of the fact that BHI and TSB already contained substantial amounts of glucose, namely 11 and 14 mM, respectively. Interestingly, supplementation with 0.2% glucose (10 mM) was sufficient to induce a visible biofilm (A_{530}, >1.5) in all three media tested, and a further increase in glucose concentration up to 1% only slightly increased biofilm formation. In contrast to the findings of Beenken et al. (1), who observed biofilm formation of SA113 in polystyrene microtiter plates only in media that were supplemented with both sodium chloride (3%) and glucose and when the wells of the microtiter plates were precoated with plasma proteins, neither addition of 3% salt to the growth medium nor precoating the microtiter plates with plasma proteins was essential for, or increased biofilm formation of SA113 in our experiments, suggesting that SA113 strains with altered adhesion/biofilm-forming capacities may exist in different laboratories.

Characteristics of the SA113 biofilm. The biofilm produced by SA113 in TSB supplemented with 1% glucose was sodium metaperiodate, proteinase K, and DNase I sensitive (Fig. 1B), indicating the presence of PIA, proteinaceous factors, and genomic eDNA. This is in line with previous observations (13) and supports the assumption made by Rohde et al. (46) that biofilm formation depends on protein factors in addition to PIA. Moreover, it showed that DNA was also an important structural component of the biofilm formed by this strain.

When strain SA113 was grown in glass tubes in TSB supplemented with 1% glucose, cells clumped together and sank to the bottom of the tube, leading to a clearance of the medium (Fig. 2). We analyzed this effect in the *ica*-negative strain SA113 Δ*ica* (13), which remained cloudy after growth in glass tubes in TSB supplemented with 1% glucose (Fig. 2), indicating, that the clumping might be due to PIA production. Interestingly, addition of DNase I (140 U/ml) to the growth medium suppressed the clumping as well (data not shown), suggesting that clumping requires the simultaneous presence of PIA and eDNA.

Effect of CcpA on biofilm formation. Since glucose supplementation promotes biofilm formation, we analyzed the role of the catabolite control protein A (CcpA) in this process. We deleted *ccpA* in the biofilm-forming strains SA113 and DSM 20231, yielding strains KS66 and KS157, respectively, and analyzed the capacity of the mutants to form a biofilm. The growth rates of the wild type and Δ*ccpA* mutants were similar. For demonstration of biofilm formation of strain DSM 20231, the plates had to be precoated with 20% human plasma. The deletion of *ccpA* significantly reduced the biofilm formation capacity of the mutants under the respective static conditions (Fig. 3A [only SA113 shown]), and complementation of the Δ*ccpA* mutants in *trans*, using pMST1, which contains the wild-

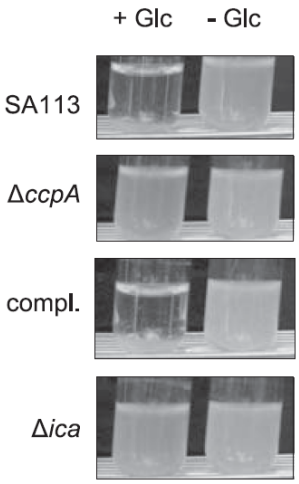

FIG. 2. Growth phenotype of SA113, KS66 (Δ*ccpA*), KS66 *trans*-complemented with plasmid pMST1 (compl.), and SA113 Δ*ica* (Δ*ica*) grown for 18 h at 37°C in glass tubes in TSB (− Glc) or TSB supplemented with 1% glucose (+ Glc).

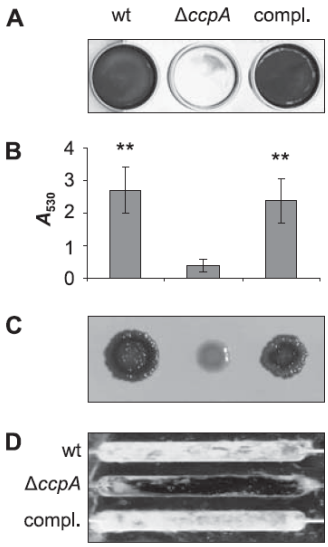

FIG. 3. (A) Effect of the *ccpA* deletion on biofilm formation capacity of *S. aureus* strain SA113 in TSB supplemented with 1% glucose. wt, wild type. (B) Quantification (A_{530}) of biofilm formation of SA113. Results represent the averages of at least three independent experiments. Error bars indicate the standard deviation of the mean. **, $P < 0.01$ for wild type and KS66 *trans*-complemented with plasmid pMST1 (compl.) versus Δ*ccpA*. (C) Growth morphologies of strain SA113 on CRA plates. (D) Biofilm formation of strain SA113 under flow conditions.

type *ccpA* allele, restored the biofilm formation capacities of both mutants to wild-type levels (Fig. 3A [only SA113 shown]). Transformation of the mutants with the empty control vector had no effect (data not shown), signaling that the decrease in biofilm formation observed in the Δ*ccpA* mutants was due to the deletion of *ccpA*. Biofilm formation for SA113 was further analyzed by quantifying biofilm formation under static conditions and by observing biofilm formation under flow cell conditions (Fig. 3B and D). Both experiments confirmed the reduced capacity of the mutant to form biofilm. Inactivation of *ccpA* had no apparent effect on the primary attachment of the mutant to polystyrene surfaces. Both the wild type and mutant showed approximately 10% primary adherence (data not shown). Interestingly, the lack of *ccpA* in SA113 did suppress the clumping phenotype in the presence of glucose observed for the parental strain grown in glass tubes (Fig. 2). The Δ*ccpA* mutant also lost the ability of its parent to form black and crusty-appearing colonies on CRA plates (Fig. 3C) and formed smaller colonies on CRA. The reason for the latter phenomenon might be the slightly higher susceptibility of the mutant (MIC of 1 g/liter versus 2 g/liter for Congo red), which was in the range of the Congo red concentration in the agar (0.8 g/liter). The crustiness and black color on CRA have been associated with PIA production (29) and might therefore indicate that the Δ*ccpA* mutant of strain SA113 produced less PIA than the wild type. *trans*-Complementation of KS66 with pMST1 restored both the colony morphology on CRA plates and the clumping phenotype of SA113 cultures grown in glass tubes, indicating that CcpA was involved in the development of both phenotypes.

Effect of CcpA on *ica* expression and PIA production. To support our proposed effect of CcpA on PIA production, we quantified the *icaA* transcription and determined the PIA production of SA113 and its Δ*ccpA* mutant KS66 after 2 h (early exponential growth phase) and 8 h (stationary phase) of growth. Monitoring the *icaA* transcription after 2 h of growth in TSB supplemented with 1% glucose revealed a clear difference between SA113 and KS66, with SA113 producing 0.113 ± 0.006 copy of *icaA* per copy of *gyrB*, while deletion of *ccpA* resulted in a sevenfold reduction in *icaA* transcription (0.015 ± 0.004 copy of *icaA* per copy of *gyrB* for KS66), as compared to the wild type ($P < 0.01$). *trans*-Complementation of KS66 with pMST1 strongly increased *icaA* transcription (0.47 ± 0.144 copy of *icaA* per copy of *gyrB*), while transformation of KS66 with the empty control plasmid pAW17 did not alter the *icaA* expression of the mutant (0.018 ± 0.002 copy of *icaA* per copy of *gyrB*) (Fig. 4 A). Surprisingly, after 8 h of growth, *icaA* transcript levels were found to be strongly reduced and no longer differed significantly between SA113 and KS66 (0.001 ± 0.0004 copy of *icaA* per copy of *gyrB* for SA113 and 0.004 ± 0.004 copy of *icaA* per copy of *gyrB* for KS66; $P > 0.05$). Analysis of the PIA production in SA113 and its Δ*ccpA* mutant KS66 identified significant amounts of PIA only in SA113 but not in KS66 (Fig. 4B). In line with our transcriptional data, PIA was only detectable during the early exponential growth phase (2 h), but not after 8 h of growth (data not shown). *trans*-Complementation of KS66 with pMST1 restored PIA production, while transformation of KS66 with pAW17 was found to have no effect, signaling that CcpA is indeed influencing *ica* transcription and PIA production in SA113.

Effect of CcpA on TCA cycle genes. Based on the observations of Vuong and coworkers (56a), who found that decreased tricarboxylic acid (TCA) activity was associated with increased PIA production in *S. epidermidis*, and on the findings of Kim et al. (28), showing that transcription of the genes for the TCA

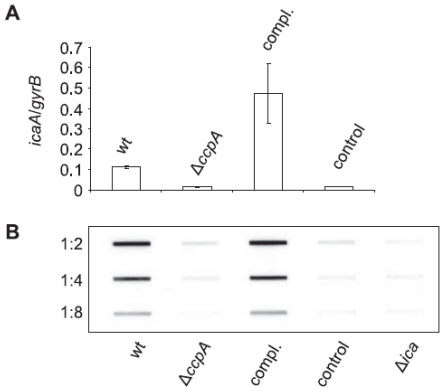

FIG. 4. (A) *icaA* transcription after 2 h of growth in TSB supplemented with 1% glucose. (B) Slot blot analysis of the PIA production after 2 h of growth in TSB supplemented with 1% glucose. PIA extracts were diluted as indicated. wt, wild-type strain SA113; Δ*ccpA*, strain KS66; compl., strain KS66 complemented with pMST1; control, strain KS66 containing pAW17.

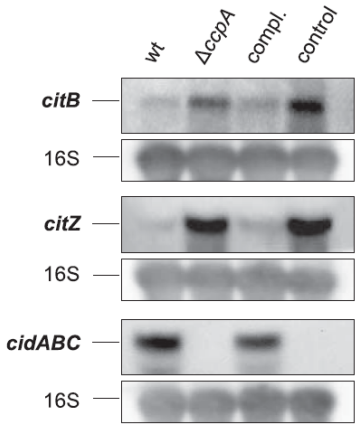

FIG. 5. Northern blot analysis of *citB*, *citZ*, and *cidABC* transcription after 2 h of growth in TSB supplemented with 1% glucose. A 16S rRNA gene probe was used for hybridization as a loading control. wt, wild-type strain SA113; Δ*ccpA*, strain KS66; compl., strain KS66 complemented with pMST1; control, strain KS66 containing pAW17.

cycle enzymes CitB (aconitase) and CitZ (citrate synthase) are influenced by CcpA in *Bacillus subtilis*, we monitored the effect of CcpA on the transcription of *citB* and *citZ* in *S. aureus* under biofilm inducing conditions. Similar to the findings of Vuong et al. (56a) and Kim et al. (28), we found a strong increase in *citB* and *citZ* transcription in KS66 during the early exponential growth phase (Fig. 5), and *trans*-complementation of the Δ*ccpA* mutant with pMST1 reduced *citB* and *citZ* transcription to levels seen in the wild type. We found a putative *cre* (catabolite-responsive element) site 27 bp upstream of the *citZ* open reading frame (TgTGAAAGCcATTTCATA; capital letters indicate nucleotides that correspond to the *cre* site consensus of *B. subtilis* [38]), suggesting that CcpA affects the transcription of *citZ* directly. The effect of CcpA on *citB* expression, on the other hand, appears to be indirect because such an element is missing in front of, or within, *citB*, similar to the situation found in *B. subtilis* (28).

Effect of CcpA on the transcription of selected factors known to be involved in the regulation of biofilm formation. A series of genetic loci have been identified to influence the primary attachment and/or the cellular accumulation process in *S. aureus* in addition to the *ica* operon (reviewed by O'Gara [39]) and Tu Quoc et al. [55]). Our special interest focused on the impact of CcpA on the transcription of (i) *rbf*, due to its demonstrated influence on biofilm formation in response to glucose and salt (32); (ii) *sarA*, since the inactivation of *sarA* was shown to abolish biofilm formation in SA113 (1); (iii) *atl* and *cidA*, since they were shown to contribute to DNA release and biofilm development (4, 26, 44); (iv) *mgrA*, an important regulator of autolysis, which has recently been shown to be involved in biofilm formation (55); and (v) *arlRS*, another regulator of autolysis and cell division (21), since our computational analysis identified a putative *cre* (AATtTAAACGTA AACAAA; capital letters indicate nucleotides that correspond to the *cre* consensus of *B. subtilis* [38]) 95 bp downstream of the *arlRS* transcriptional start site (21a). We therefore analyzed the impact of CcpA on the transcription of *arlRS*, *atl*, *mgrA*, *rbf*, *cidABC*, and the *sarA* locus by monitoring the expression of these genes during the early exponential growth phase by Northern blot analysis. These assays revealed no apparent differences in *arlRS*, *mgrA*, *rbf*, or *sarA* transcription between the wild-type strain and its corresponding Δ*ccpA* mutant, suggesting that CcpA is not affecting the regulation of these genes (data not shown). The investigation of the effect of CcpA on *atl* transcriptional levels suggested a tendency toward higher *atl* expression in the wild type than in the mutant, though total amounts of transcripts varied widely between separate experiments (data not shown).

However, we found a clear effect of CcpA on *cidABC* transcription, with SA113 producing significant amounts of *cidABC*, while no transcript was observed in KS66 (Fig. 5), and *trans*-complementation of the Δ*ccpA* mutant with pMST1 led to the production of *cidABC* transcripts again. The acetic acid concentration at the time point of sampling for Northern blot analysis was 2 mM in the culture supernatant of both the wild type and mutant. Because the acetic acid concentration was lower than the concentration required for *cidA* induction (30 mM) according to reference 45, and the concentrations were identical in the wild type and mutant, we suggest that there may exist additional factors which can induce *cidA* transcription. As mutation of *cidA* has been associated with reduced autolysis (41), we analyzed the effect of the *ccpA* mutation on Triton X-induced autolysis. The wild type showed slightly faster autolysis, with 88% ± 9.0% of the cells lysed after 2 h, while the mutant showed only 66% ± 3.7% lysis at this time point. When *trans*-complementing the mutant with pMST1, lysis was partially restored to 72% ± 6.3% after 2 h.

Conclusion. The rapid adaptation to environmental and nutritional conditions is central to the success of *S. aureus* as pathogen. The utilization of glucose as the preferred carbon source is controlled in *S. aureus* by CcpA, the mediator of carbon catabolite repression, which was shown earlier to promote the expression of selected virulence factors, increase the expression of oxacillin resistance, and repress capsule synthesis (50). The role of CcpA as a mediator of biofilm formation in

the presence of glucose adds a further important function to CcpA, contributing to staphylococcal virulence and persistence. The positive impact of CcpA on biofilm formation in *S. aureus* is partly in contrast to observations made in *B. subtilis* (7, 53), where CcpA, depending on the growth medium, was found to repress biofilm formation. However, biofilm formation in *S. aureus* differs widely from biofilm formation in *B. subtilis* (8), which in the latter bacterium is closely related to sporulation. The *S. aureus* biofilm was shown here to have a complex composition, including PIA, proteinaceous factors, and eDNA. CcpA had an impact on at least two of these components: through upregulation of the *ica* operon, which is involved in PIA synthesis; and through upregulation of *cidA*, coding for the holin CidA, postulated to be involved in the release of eDNA. Regulation of these genes, which are not accompanied by a *cre* site, may have occurred indirectly, as a consequence of the downregulation of the TCA cycle through repression of *citB* and *citZ*, with *citZ* being preceded by a typical *cre* consensus sequence recognized by CcpA. These findings confirm the reported role of downregulation of the TCA cycle in biofilm formation (28). The apparent correlation between oxacillin resistance and the capacity to form a biofilm, as observed in some methicillin-resistant *S. epidermidis* strains (10), exists also in *S. aureus* and may be linked over CcpA.

Inactivation of CcpA in *S. aureus*, incapacitating biofilm formation, reducing the expression of secreted virulence factors and lowering the expression of oxacillin resistance, makes it an important player in overall staphylococcal virulence and antibiotic resistance. These findings underline the important linkage of metabolism to the envelope composition, virulence, and resistance in *S. aureus*.

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3.3 Project 3

Staphylococcus aureus CcpA controls *tst* expression

Kati Seidl[1], Markus Bischoff[1,2], and Brigitte Berger-Bächi[1]

[1]Institute of Medical Microbiology, University of Zürich, Zürich, Switzerland
[2]Institute of Medical Microbiology and Hygiene, University of Saarland Hospital, Homburg/Saar, Germany

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ABSTRACT

The production of the *Staphylococcus aureus* toxic shock syndrome toxin TSST-1, encoded by the *tst* gene, is influenced by several regulators, and is subject to modulation by environmental factors such as pH, aerobiosis and glucose. We show here that the glucose-induced repression of *tst* is mediated by the catabolite control protein CcpA. Computational analysis identified potential binding-sites for CcpA in the promoter regions of all sequenced *tst* genes. These so called catabolite responsive elements (*cre*) were identical, except that of the bovine mastitis isolate RF122, which differed by two base pairs, but was still palindromic to the same degree. *tst*-promoter reporter gene fusion assays using the *tst* promoter of strain N315 indicated that already 1 mM glucose repressed *tst* transcription. In a Δ*ccpA* background, no such repression could be observed, suggesting that CcpA was the mediator of the glucose-mediated repression. The *tst* genes of strains N315 and RF122, representing both *cre*-variants were cloned on a plasmid under their native promoters and introduced into strain Newman and its isogenic Δ*ccpA* mutant. Glucose strongly repressed transcription of both *tst* genes in strain Newman, whereas glucose repression was abolished in the isogenic Δ*ccpA* mutant, confirming the data obtained from the reporter gene fusion assays. Glucose repression of both, *tst* transcription as well as TSST production was also confirmed in the TSST-1 producing RF122. In contrast, the *tst* encoding strain N315 was found to be a very poor TSST producer with a low and seemingly glucose independent *tst* transcription.

INTRODUCTION

Toxic shock syndrome (TSS) is an acute and potentially fatal illness, which is caused by a group of bacterial superantigens, such as toxic shock syndrome toxin (TSST-1), staphylococcal enteroxins and streptococcal pyrogenic toxins. Superantigens, unlike conventional peptide antigens, bind to invariant regions of the major histocompatibility complex class II molecules at the surface of antigen-presenting cells outside of the classical antigen-binding groove and also to invariant regions of the T-cell receptor. This leads to activation of T-cells at orders of magnitude above antigen-specific activation, resulting in massive cytokine release, which in turn leads to capillary leakage and is believed to be responsible for hypotension, shock, and finally death (17). Especially in the early 1980's, there was a major interest in TSS, because it was reported to be the cause of several staphylococcal TSS cases in otherwise healthy young women (7). This rise in TSS cases was associated with high-absorbency tampons and the disease was named menstrual TSS. Menstrual TSS is caused by TSST-1, which is produced by *S. aureus* and is encoded by *tst,* a gene found on several staphylococcal pathogenicity islands (SaPIs) (reviewed in (22)).

The regulation of TSST-1 is known to be complex and several environmental conditions, including salt, oxygen and carbon dioxide, growth rate, temperature, glucose and pH were reported to modulate its production (3, 26, 28, 40). Global regulators shown to be involved in the regulation of TSST-1 are SarA (4, 6, 21), *RNAIII* of the *agr* system (3, 25), and SrrAB (39) (Fig.1). However, there is some contradiction about the role of SarA in *tst* expression, since Chan and Foster (4) described that repression of *tst* by SarA was *agr*-independent, while Novick (21) found that *tst* repression was dependent on *agr*. SrrAB, which is also a repressor of *RNAIII* (24), was previously shown to repress *tst* transcription under limited oxygen pressure (39). More recently, Pragman et *al*. (23) proposed that SrrAB, in addition to its downregulatory function under low-oxygen conditions, enhances the levels of *tst* under aerobic conditions. More complexness is added by the fact, that *tst* has autoregulatory functions, with TSST-1 acting as a repressor on *tst* transcription (35) (Fig. 1).

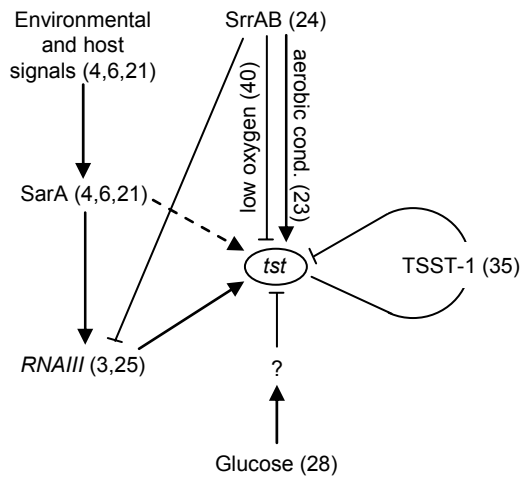

Figure 1: Regulation of *tst* transcription. Arrows represent upregulation, bars represent downregulation, dashed arrows indicate controversial findings, and numbers in brackets indicate references. ?, CcpA (this study).

We recently analyzed the impact of the carbon catabolite protein A (CcpA) of *S. aureus* on carbon metabolism, virulence determinant expression and biofilm formation (29, 30). CcpA induces or represses the transcription of genes by binding to so-called catabolite responsive elements (*cre*). These *cis*-acting DNA sequences, which consist of 14 to 18 base pairs, have been extensively studied in *B. subtilis* (15, 18, 19, 32, 38, 41). Whether the transcription of a gene is repressed or activated by CcpA depends on the location of the *cre* relative to the transcriptional start site. When the *cre* is located upstream of the -35 region activation occurs (34), location in the promoter or coding region region on the other hand leads to prevention of transcription initiation (10) or blockage of the elongation (42). Binding of CcpA to the *cre*-sites is induced by complex formation with HPr, a component of the phosphoenolpyruvate-dependent phosphotransferase transport system (37). In the presence of glucose or other rapidly metabolized carbon sources, HPr is phosphorylated on serine-46, promoting it to bind to CcpA. But CcpA can apparently also regulate gene expression without binding to *cre*-sites (11, 12, 20). It was proposed that CcpA can directly interact with and inhibit RNA polymerase, a process that is stimulated by NADP or NADPH, independently of the redox state of the cell (12).

Since TSST-1 production was shown to be repressed by glucose (28), and as the *tst* promoter region contained a *cre*-site, we investigated whether CcpA was the mediator of this regulatory phenomenon. We therefore analyzed the effect of glucose on *tst* transcription and TSST-1 production and compared it to the effect in a *ccpA* null mutant. Our results suggest that repression of *tst* by glucose is directly mediated by the carbon catabolite protein CcpA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains, relevant phenotypes and plasmids used in this study are listed in Table 1. All strains generated for this study were confirmed by pulsed field gel electrophoresis of total genome SmaI digests (36) and by Southern blot analysis according to standard protocols using a CcpA-specific DIG-probe which was generated using primers previously published (30). *S. aureus* was routinely grown in Luria-Bertani (LB) medium buffered with 50 mM HEPES (pH 7.5) with a flask volume/culture volume ratio of 5:1 at 37°C and 200 rpm. Media were supplemented with 50 µg ml^{-1} kanamycin, if appropriate.

Table 1: Strains and plasmids used in this study

	Relevant genotype and phenotype	Source or reference
S. aureus		
RN4220	NCTC8325-4 r^- m^+ (restriction minus, modification plus)	(13)
Newman	ATCC25904; clinical isolate, CP5 producer	(8)
MST14	Newman *ccpA::tet*(L), Tc^r	(30)
N315	Multiresistant clinical MRSA isolate, TSST-1-producer	(14)
RF122	Wild type strain from bovine mastitis, TSST-1-producer	(9)
KS87	Newman pSKA12 ($tstp_{N315}$*::luc*$^+$), Kan^r	This study
KS64	Newman *ccpA::tet*(L) pSKA12 ($tstp_{N315}$*::luc*$^+$), Tc^r, Kan^r	This study
KS179	Newman pSKA20 ($tst_{N315}{}^+$), Kan^r	This study
KS180	MST14 pSKA20 ($tst_{N315}{}^+$), Tc^r, Kan^r	This study
KS181	Newman pSKA21 ($tst_{RF122}{}^+$), Kan^r	This study
KS182	MST14 pSKA21 ($tst_{RF122}{}^+$), Tc^r, Kan^r	This study
E. coli		
DH5α	Restriction-negative strain for cloning	Invitrogen
Plasmids		
pSP*luc*+	Luciferase fusion plasmid, ColE1 replication origin, Ap^r	Promega
pCN34	*E. coli* - *S. aureus* shuttle plasmid, ColE1 replication origin and pT181 *cop-wt repC*, Amp^r, Kan^r	(5)
pAW17	*E. coli* - *S. aureus* shuttle plasmid, ColE1 and pAMα1 replication origins, Kan^r	(27)
pSKA12	pCN34 containing a 2.5-kb $tstp_{N315}$*::luc*$^+$ fusion, Ap^r, Kan^r	This study
pSKA20	pAW17 with a 1-kb PCR fragment covering *tst* of strain N315 and its proposed promoter, Kan^r	This study
pSKA21	pAW17 with a 1-kb PCR fragment covering *tst* of strain RF122 and its proposed promoter, Kan^r	This study

Abbreviations are as follows: Tc^r, tetracycline resistant; Kan^r, kanamycin resistant; Ap^r, ampicillin resistant.

Construction of plasmid *tstp*$_{N315}$*::luc*+-pCN34 (pSKA12). A 0.8 kb fragment containing the *tst* promoter region was amplified by PCR from chromosomal DNA of *S. aureus* N315 by the use of primers TstPAsp718+ (GCGCCATGGTTAATTCTCCTTCATTCAAA) including an Asp718 linker (underlined) and TstPNcoI- (GCGGGTACCTTCGAGAGGCAGATTACTCC) including a NcoI linker (underlined). The PCR product was cloned in front of the luciferase gene of plasmid pSP*luc*+. Therefrom, a 2.4 kb Asp718-EcoRI fragment, including the *tst* promoter region fused to the luciferase coding region, was cloned into plasmid pCN34, generating plasmid pSKA12. The identity of the construct was confirmed by sequence analysis and comparison to the respective N315 sequence of the NCBI database. The plasmid was electroporated into RN4220 and subsequently transduced into strain Newman and its isogenic Δ*ccpA* mutant (MST14), generating strains KS87 and KS64, respectively.

Construction of plasmids *tst*$_{N315}$-pAW17 (pSKA20) and *tst*$_{RF122}$-pAW17 (pSKA21). 1-kb fragments containing the *tst* genes and promoter regions were amplified by PCR from chromosomal DNA of *S. aureus* N315 and RF122 using primers TstXbaI+ (GTTGTCTAGAACTCACACTTTGTTTTTTGC) including an XbaI linker (underlined) and TstPstI- (GTTGCTGCAGGTTTACTAATTCACCCTAGC) including a PstI linker (underlined). The PCR products were cloned into pAW17, generating pSKA20 and pSKA21, respectively. The identity of the constructs was confirmed by sequence analysis and comparison to the respective sequences of the NCBI database. The plasmids were electroporated into RN4220 and subsequently transduced into strains Newman and the respective Δ*ccpA* mutant (MST14), generating strains KS179, KS180, KS181 and KS182, respectively.

Luciferase assays. For glucose impulse experiments, the cultures were inoculated to an OD_{600} of 0.05 and were grown for 5 h without glucose. At 5 h, corresponding to the early stationary growth phase, the cultures were split and different amounts of glucose were added. Samples were taken at 0, 30 and 60 min after the glucose impulse. To follow the *tst*-promoter-luciferase-reporter activity over time, the medium was inoculated with an overnight culture to an OD_{600} of 0.05 and the bacteria were grown for 8 h. The medium contained either 10 mM of glucose or no glucose. Sampling was performed on an hourly basis, starting 2 h after inoculation. Luciferase activity was measured as described earlier, using a luciferase assay substrate and a Turner Designs TD-20/20 luminometer (Promega) (1). All luciferase assays were performed at least three times on independent experiments.

Northern blot analysis. Sampling for Northern blot analysis was basically performed as for the glucose impulse experiments in the luciferase assays. 10 mM glucose was added to one half of the culture, while the other half remained without glucose. Samples were centrifuged

for 2 min at 12,000 x g and cell-sediments snap-frozen in liquid nitrogen. RNA isolation and Northern blotting were performed as described earlier (16). Primers DIGtst+ (TAAGCCTTTGTTGCTTGCG) and DIGtst- (CACTTTGATATGTGGATCCG) were used to generate digoxigenin-labeled *tst* probes. All Northern blot analyses were performed at least twice in independently isolated RNA samples.

Western blot analysis. Extracellular proteins were determined from supernatants of cultures grown for 3 or 5 h either with or without 10 mM glucose. Supernatants corresponding to a final OD_{600} of 5 (OD_{600} 25 for strain N315), were adjusted to the same volume for all samples using sterile medium. Identical amounts of BSA were added to samples as an internal control for semi quantitative determination of TSST-1. Proteins were precipitated with TCA at a final concentration of 10 %, samples were iced for 30 min, centrifuged for 15 min at 16,000 x g and sediments were washed twice with 5 ml acetone. After air drying, sediments were resuspended in 200 µl loading buffer. 10 µl of sample, corresponding to a final OD_{600} of 0.25 (1.25 for strain N315) were analyzed by 10 % SDS/PAGE. Gels were either stained with Coomassie brilliant blue or blotted on nitrocellulose membranes. TSST-1 detection was performed using rabbit polyclonal anti-TSST-1 antibodies (LucernaChem, Switzerland) after blocking with human IgG and visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, US).

RESULTS AND DISCUSSION

The *tst* promoter region contains a *cre*. Screening strain N315 with the consensus sequence proposed by Miwa *et al.* (19), we identified a putative *cre*-site 22 bp upstream of the translational start of *tst* on SaPIn1 (Fig. 2A). As *tst* is located on different pathogenicity islands (SaPIs) in different strains (22), we compared the promoter regions of the *tst* genes of the published SaPIs and found identical *cre*-sites on SaPI1, SaPIm1, SaPIn1, and SaPI2 (Fig. 2B). With the exception of one base pair, the *cre*-sites are palindromic and are located downstream of the transcriptional start site suggested by Vojtov *et al.* (35). The pathogenicity island SaPIbov1 of the bovine mastitis isolate RF122 had a *cre*-site, which differed in two base pairs from the *cre*-site of the other *tst* genes. However, it was still palindromic with the exception of one base pair (Fig. 2B). The presence of *cre*-sites in the different *tst* genes together with previous findings showing that glucose decreased TSST-1 production (28) indicated that CcpA might mediate glucose-induced *tst* repression.

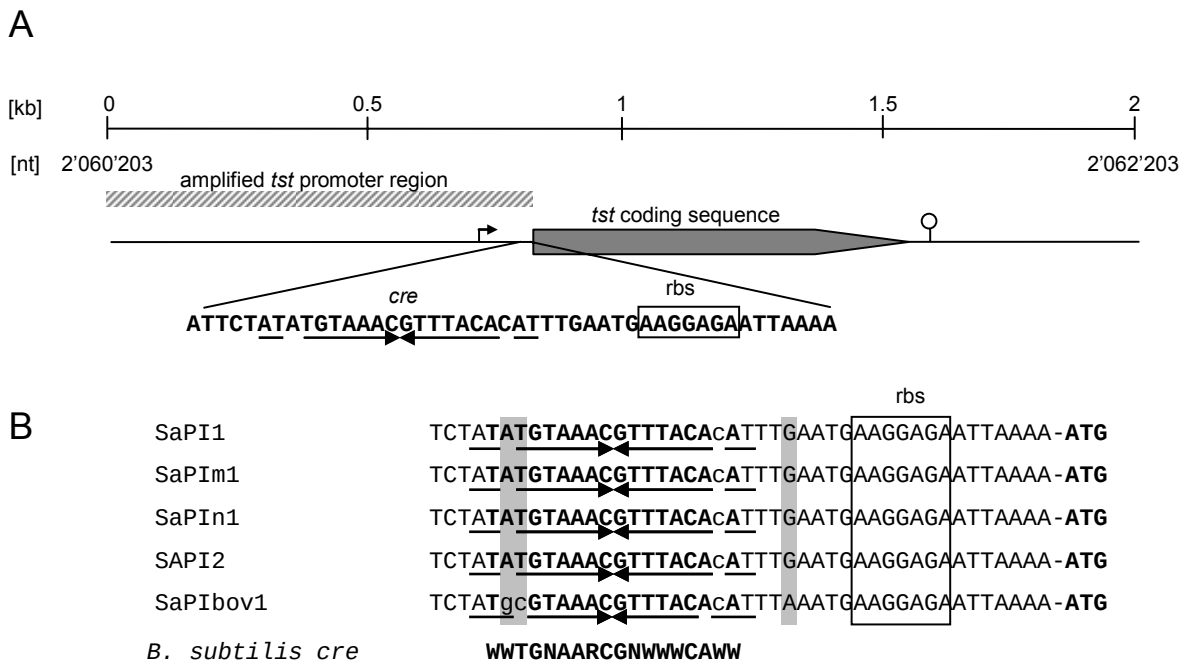

Figure 2: (A) Schematic representation of the *tst* promoter region of SaPIn1 (strain N315, accession Nr. NC_002745) showing the position of the *cre*-site. *tst* coding region, potential transcriptional start site and terminator are indicated. (B) Alignment of the *cre*-sites and their surroundings in the *tst* promoter regions of the different pathogenicity islands. Nucleotides fitting with the *cre* consensus of *B. subtilis* are highlighted in bold type. Inverted repeats are indicated by arrows. Differences in nucleotides are highlighted by grey boxes. Ambiguity codes are as follows: W denotes A or T, R denotes G or A, and N denotes A, C, G, or T. rbs: ribosome binding site as suggested by (2).

***tst* expression is strongly repressed in the presence of glucose.** To confirm the effect of glucose on the expression of *tst*, which has been previously described (28), we performed Northern blot analysis of a glucose impulse experiment. To include the two different *cre*-sites in the study, we chose strains N315 and RF122 for this purpose. As *tst* was known to be

expressed in early stationary growth phase (33), we cultured the cells in HEPES-buffered LB medium for 5 h, split the cultures and added glucose to one half.

We found a strong *tst* transcription in RF122 during this growth stage and the addition of glucose lead to the repression of *tst* (Fig. 3A). Surprisingly, in strain N315, these effects were not seen. *tst* expression was much weaker, and instead of a distinct band, smears appeared. However, strain N315 was previously reported to have a defective *RNAIII* production (31), which might explain the low *tst* transcription levels. To test the impact of the genetic background on the expression of this gene, we fused the promoter region of the *tst* gene of strain N315 to the firefly luciferase gene, yielding *tstp*$_{N315}$*::luc*$^{+}$-pCN34 (pSKA12) and transduced the construct into strain Newman. In the latter strain, *tst* promoter activity was strongly repressed by glucose (Fig. 3B), indicating that the lack of glucose-dependent repression of *tst* in N315 was likely to be due to the genetic background.

To see how much glucose would be needed for repression, we performed a glucose impulse experiment with increasing amounts of glucose. We observed that the addition of 1 mM (0.018 %) glucose reduced the luciferase activity to 66 % (Fig. 3B), 2 mM (0.036 %) glucose decreased the activity to 35 % and concentrations of more than 4 mM to less than 20 %. This indicated that transcription of the *tst* promoter of N315 was highly sensitive to low concentrations of glucose.

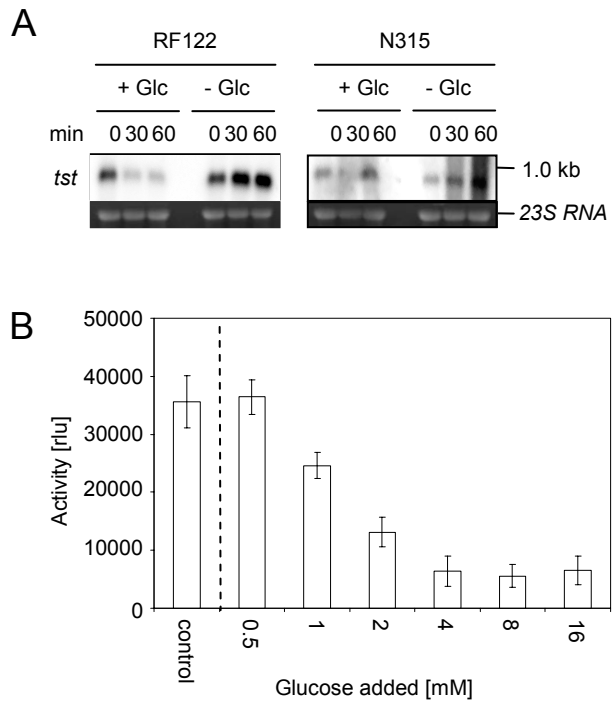

Figure 3: (A) *tst* expression in response to glucose in strains N315 and RF122. Cells were grown in HEPES-buffered LB to early stationary-growth phase (5 h, A_{600} ~ 3), cultures were split in half, and 10 mM glucose was added to one half (+ Glc) while the other half was left unchanged (− Glc). Samples were taken at 5 h (time 0), 30 and 60 min afterwards. Ethidium bromide-stained 23S rRNA patterns are shown as an indication of RNA loading. (B) Luciferase activity of strain KS87 (Newman containing a *tstP*$_{N315}$*::luc*+ construct) in response to different glucose concentrations. Cells were grown in LB medium containing 50 mM HEPES. After 5 h, luciferase activity was measured, the culture was split and different amounts of glucose were added. Luciferase activity was measured again 1 h after the impulse. The average of three independent measurements is shown. Control: activity at 5 h, before glucose addition.

Glucose-dependent repression of *tst* promoter activity is dependent on CcpA. To see whether CcpA might be necessary for the glucose-dependent repression of *tst* transcription, we transduced the *tstp*$_{N315}$*::luc*$^{+}$-pCN34 construct into the Δ*ccpA* mutant of strain Newman and followed the glucose-effect over time in wild type and mutant. In the wild type, repression

of luciferase activity was clearly detectable already 30 min after glucose addition, and activity decreased further until 1 h after glucose addition (Fig. 4A). After this time point, measurements were discontinued, because the pH could not be kept stable any longer, and changes in pH are known to affect *tst* expression (28). In the mutant, no changes in activity were observed upon glucose addition, strongly suggesting that CcpA was indeed involved in glucose-mediated *tst* repression (Fig 4B). Interestingly, total activity before glucose addition was weaker in the wild type than in the mutant, with 35.800 (± 3.300) relative light units (rlu) and 52.700 (± 9.800) rlu, respectively, suggesting that CcpA exerted some regulatory function on *tst* expression even in the absence of glucose.

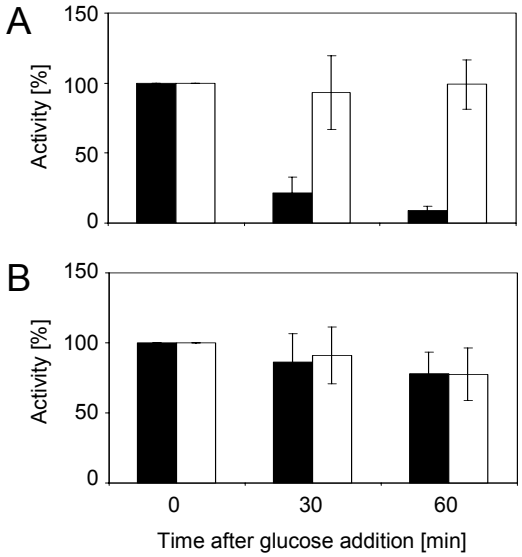

Figure 4: (A) Relative luciferase activity of strain KS87 (Newman *tstp*$_{N315}$*::luc*$^{+}$) and (B) KS64 (Newman Δ*ccpA tstp*$_{N315}$*::luc*$^{+}$) in a glucose impulse experiment. Cells were grown in LB medium containing 50 mM HEPES. After 5 h (time 0), the culture was split and 10 mM glucose were added to one half of the culture, (black bars); while the other half remained without glucose, (white bars). Activity at time point 0 was set to 100 % and relative activity after 30 and 60 min was assessed. The average of three independent measurements is shown. Total activity at time 0: wt, 35.800 (± 3.300); mut, 52.700 (± 9.800).

To observe long-term effects of glucose on *tst*-promoter activity during growth, wild type and mutant were grown for 8 h either in the presence or absence of glucose, and luciferase activity was followed on an hourly basis. Without glucose, activity in the wild type started to raise after 3 h and reached values of around 40'000 rlu (Fig. 5A). In the presence of glucose, activity remained very low (300 to 600 rlu), started to rise only slowly after 5 h, when glucose was depleted from the medium, and finally reached a similar activity as when grown without glucose (Fig. 5 A). In the presence of glucose the cells grew to a higher OD_{600}, and despite HEPES buffering, the pH dropped slightly after 5 h (Fig. 5B). Promoter activity in the mutant was about the same in the presence or absence of glucose (Fig. 5A). In the mutant, the difference in OD_{600} between cells grown in the presence or absence of glucose was smaller than in the wild type, and the pH remained stable up to 7 h (Fig 5B). As seen before, promoter activity in the mutant was generally higher than in the wild type and rose faster reaching values of around 60'000 rlu.

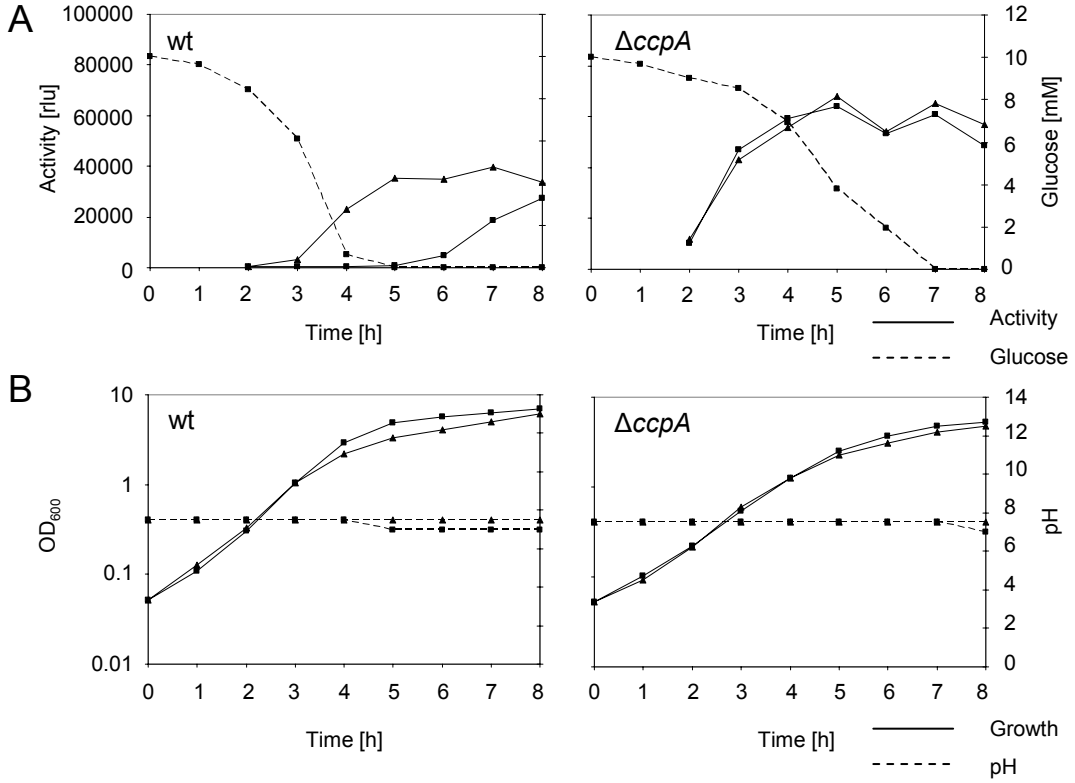

Figure 5: (A) Luciferase activity and glucose consumption; and (B) growth and pH of strains KS87 (wt), and KS64 (Δ*ccpA*); over time. Cells were grown in LB medium containing 50 mM HEPES with 10 mM glucose, (■); or without glucose, (▲). Data are representative for three independent measurements.

To test whether the lack in glucose-mediated *tst* repression in N315 might be due to an impaired CcpA activity in this strain, we investigated the glucose-mediated repression of *pckA*, a gene previously reported to be under the control of CcpA (26). As in strain Newman and RF122, *pckA* expression was strongly repressed in strain N315 upon glucose addition, indicating that this strain possessed a functional CcpA (data not shown).

The luciferase data left the question open, whether and how CcpA would repress *tst* transcription in the presence of an intact *tst* gene. Unfortunately, we were neither successful in transducing the Δ*ccpA* mutation into strain N315 nor RF122. We therefore introduced the *tst* genes of these two strains *in trans* into strain Newman and its Δ*ccpA* mutant. The genes were cloned on a plasmid and the inserts of both constructs were sequenced. *tst* transcripts were compared to transcripts of strain N315 and RF122 and were found to have identical sizes (1 kb) in all strains (Figure 6A). Transcription levels of the chromosomal tst_{RF122} of strain RF122 and strain Newman carrying the tst_{RF122} plasmid (KS181) were similar, while in the Δ*ccpA* mutant (KS182) they were weaker. A strong difference in expression was observed between chromosomal tst_{N315} of strain N315, which was very week and the plasmid borne tst_{N315} carrying derivative of strain Newman (KS179), which showed a very strong expression. Again, *tst* transcription in the Δ*ccpA* mutant (KS180) was weaker than in the wild type, but

still much stronger than in the native strain N315. TSST was reported to have autoregulatory properties (35). Interestingly, the *tst*-gene in the Δ*ccpA* mutants showed lower transcription than in the corresponding wild type, while *tst*-promoter gene fusions lacking an intact *tst* gene exhibited higher activity in the mutant than in the wild type. This observation might be in part due to *tst*-autorepression, but it could be also due to CcpA-regulation in absence of glucose. Further investigations are ongoing.

When performing glucose impulse experiments with the strains carrying the *tst*-plasmids, we found strong repression of *tst* expression by glucose in the parental strains, independently of whether they carried the N315 or the RF122 *tst* gene. No such effect was observed in the Δ*ccpA* mutants (Fig. 6B). The fact that both *tst* genes were repressed by glucose in a CcpA-dependent manner suggested that the minor difference in their *cre-site*s did not affect CcpA activity.

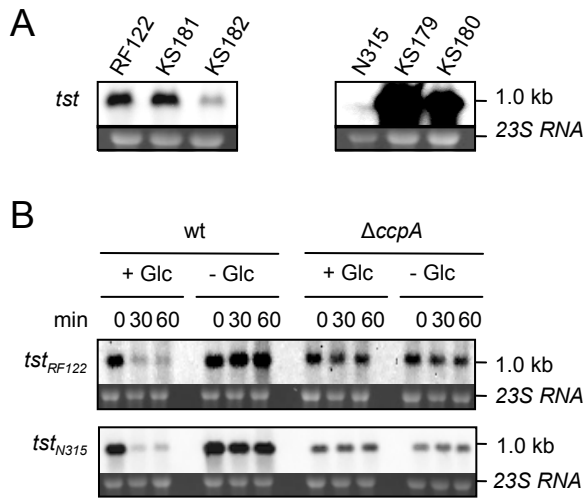

Figure 6: (A) Comparison of size and strength of the *tst* transcripts of all TSST-1 strains used in this study. Samples were taken after 5 h growth. (B) Northern blot analysis of *tst* in strains Newman (wt) and MST14 (Δ*ccpA*) carrying pSKA20 (tst_{N315}) or pSKA21 (tst_{RF122}). Cells were grown in HEPES-buffered LB to early stationary-growth phase (5 h, A_{600} ~ 3), cultures were split in half, and 10 mM glucose was added to one half (+ Glc) while the other half was left unchanged (− Glc). Samples were taken at 5 h before glucose addition (time 0), 30 and 60 min afterwards. The expression of tst_{N315} had to be adjusted in comparison to tst_{RF122}. Samples were loaded as indicated. Ethidium bromide-stained 23S rRNA patterns are shown as an indication of RNA.

The question remains open, why strain N315 exhibits such a weak tst_{N315} expression that is insensitive to glucose-induced stimuli, although this strain seems to possess normal CcpA activity. We assume that the lack of *RNAIII* in N315 (31), which was previously shown to be an important inducer of *tst* transcription (3, 25), may be responsible for the low level of *tst* transcription in this strain. The remaining basal *tst* expression in strain N315 might be either too low to see a CcpA-mediated repression or it might be insensitive to this type of regulation. As N315 also differs in terms of metabolism from the other strains by being unable to catabolize acetate in the post-exponential growth phase (31), this loss of secondary metabolite catabolism might have consequences on *tst* expression as well. Alternatively, sequence differences between strain RF122 and N315 upstream of the cloned promoter regions might be the reason for additional regulatory or autoregulatory effects and thus for the different expression levels. Also, regulatory circuits which might be present in strain N315, but not in the other strains could be the reason for the low *tst* expression in this strain. To answer all these questions, further investigations should be carried out.

***tst* does not affect exoprotein patterns of strain Newman.** To asses how much TSST-1 was produced by the different strains, we analyzed supernatants of post exponential phase cultures. Figure 7A shows the amount of TSST-1 in the supernatant after five 5 h growth in all strains with strain Newman serving as negative control. Consistent with its low *tst*-transcription, strain N315 produced smaller amounts of TSST-1 than the other strains. Unlike Vojtov and Novick (35), who found a strong difference in exoprotein pattern between TSST-1-producing isolates and their isogenic non-TSST-1-producing derivatives, we did not observe significant differences in the exoprotein patterns of strain Newman and its TSST-1-producing derivatives (Fig. 7B). Although TSST-1 gave strong signals in Western blot analysis, the band could not be seen on Coomassie stained gels, indicating that the TSST-1 band might be masked by comigrating proteins or might be present at low levels.

As we used the same forward primer as Vojtov and Novick had used to clone the *tst* gene, we can exclude, that differences in the cloned promoter region account for this discrepancy. However, Vojtov and Novick used the *tst*-gene of SaPI1, which slightly differs in the amplified promoter region through the lack of two thymidines 141 bp upstream of the translational start. Also the use of different genetic backgrounds might account for the different findings.

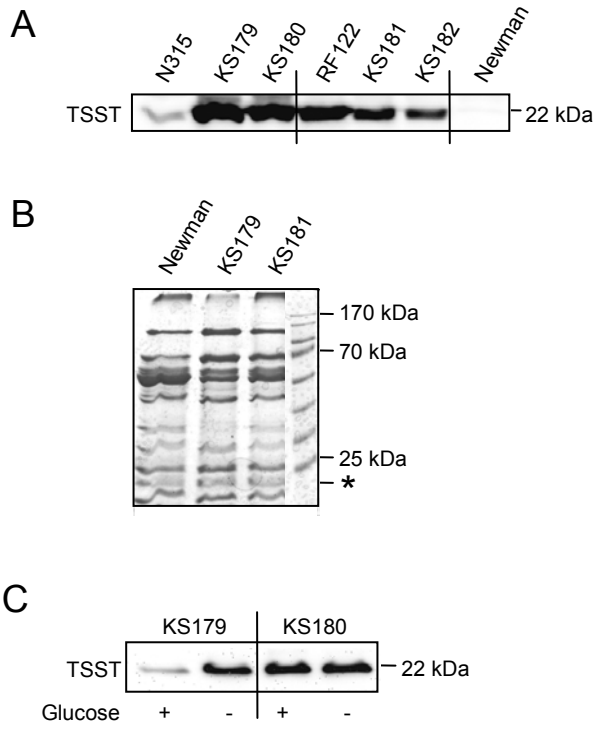

Figure 7: (A) Comparison of TSST-1 amount after 5 h in the culture supernatants of all TSST-1-producing strains used in this study. Strain Newman served as a negative control. (B) Exoprotein patterns of strain Newman, KS179 and KS181 after 5 h growth analyzed by SDS/PAGE. The gel was stained with Coomassie brilliant blue. *, position of TSST. (C) Comparison of TSST-1 production in response to glucose in strain KS179 and KS180. Cells were grown for 3 h either with (+) or without (-) glucose and extracellular proteins were precipitated. BSA was added to the supernatant and served as control for equal precipitation and loading (not shown). KS179, Newman pSKA20 (tst_{N315}); KS180, Newman Δ*ccpA* pSKA20 (tst_{N315}); KS181, Newman pSKA21 (tst_{RF122}); KS182, Newman Δ*ccpA* pSKA21 (tst_{RF122}).

Glucose affects TSST-1 production in a CcpA-dependent manner. To confirm that the observed glucose-mediated repression of *tst* transcription also affected TSST-1 production, we determined the TSST-1 content in the supernatants of KS179 (Newman tst_{N315}) and KS180 (Newman Δ*ccpA* tst_{N315}) cell cultures after 3 and 5 h of growth in the presence and absence of 10 mM glucose. In line with the transcriptional data, we found a clear decrease in

TSST-1 in the supernatant of strain KS179 grown for 3 h in the presence of glucose, while the TSST-1 levels in the supernatants of strain KS180 were almost identical after growth for 3 h in the presence or absence of glucose (Fig. 7C). Glucose also repressed TSST-1 production in strain RF122 (data not shown). Surprisingly, after 5 h of growth, this effect was no longer observed. After this period of time, KS179 cell cultures grown in the presence or absence of glucose showed nearly identical TSST-1 levels in their supernatants, irrespective of the clear differences in *tst* transcription, indicating that even low levels of *tst* transcripts were apparently sufficient to lead to the accumulation of high amounts of TSST-1. Schlievert and Blomster (28) found strong repression of TSST-1 production using 3 % (165 mM) of glucose in the stationary growth phase, but not with lower glucose concentrations. However, this effect might also be due to a drop in pH, as they also reported that TSST-1 production was pH-dependent (28).

In agreement with our transcriptional findings, strain N315 produced only low amounts of TSST-1 after 3 h of growth. Even at later time points, TSST-1 levels in the supernatants of the N315 cell cultures were still significantly lower than the TSST-1 levels found in the supernatants of other strains (Fig. 7A).

CONCLUSION

The expression of *S. aureus* virulence genes is regulated by complex networks in which global regulators such as the *agr* system and the *sarA* family play a central role. We recently showed that CcpA is also involved in the regulation of major virulence factors and regulators of virulence, influencing the expression of *hla, spa* and *RNAIII*. By showing that also the expression of *tst* is affected by CcpA, we add another important virulence factor to the group of CcpA-regulated genes/operons. Our finding that *S. aureus* basic metabolic processes and pathogenesis are intimately linked through CcpA yield enhanced knowledge of a strategy used by this pathogen to adapt virulence factor production to particular environmental conditions.

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3.4 Project 4

Analysis of the CcpA-regulon in *Staphylococcus aureus*

Kati Seidl[1], Patrice François[2], Jacques Schrenzel[2], Markus Bischoff[3] and Brigitte Berger-Bächi[1]

[1]Institute of Medical Microbiology, University of Zürich, Zürich, Switzerland
[2]Service of Infectious Diseases/Genomic Research Laboratory, University of Geneva Hospitals, Geneva, Switzerland
[3]Institute of Medical Microbiology and Hygiene, University of Saarland Hospital, Homburg/Saar, Germany

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ABSTRACT

CcpA is the main regulator of carbon catabolite repression and activation in low-GC Gram positive bacteria. Though extensively studied in other bacteria, especially in the model organism *Bacillus subtilis*, the CcpA regulon has never been investigated in the pathogen *S. aureus*. In this study, we analysed the transcriptomes of *Staphylococcus aureus* strain Newman and its Δ*ccpA* mutant in response to glucose during the exponential growth phase. By buffering the medium, secondary effects due to acidification of the medium in response to excretion of overflow metabolites were avoided. In the presence of glucose, CcpA was found to regulate genes coding for metabolism, but also for virulence determinants. More genes were found to be differentially expressed by CcpA in a glucose-independent manner than in the classical, glucose-dependent way, suggesting that glucose-independent regulation by CcpA might be of particular importance in *S. aureus*.

INTRODUCTION

The catabolite control protein A (CcpA), a member of the LacI/GalR family of transcriptional regulators, is found in virtually all low-GC Gram-positive bacteria (70). It has been extensively studied in the model organism *B. subtilis* (10, 39, 41, 50, 73), and has been shown to have a similar role in controlling metabolism in other bacteria, such as *Staphylococcus xylosus* (28), *Lactococcus lactis* (76), *Streptococcus pneumoniae* (27) and *Listeria monocytogenes* (6). CcpA can either be a transcriptional repressor or activator, permitting the bacteria to utilize the carbon source that allows the fastest growth rate (reviewed in (17, 65, 67)). For activity, CcpA interacts with HPr, a component of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), or a closely related protein Crh, which is missing in some low-G+C Gram-positives, such as staphylococci (17). In the presence of glucose or other rapidly metabolized carbon sources, these two proteins are phosphorylated on a specific serine residue and consequently bind independently to CcpA. The complex has a high affinity for the so-called catabolite responsive elements (*cre*) in *B. subtilis* (56). These *cis*-acting DNA sequences, have been extensively studied through mutagenesis studies (42, 46, 47, 66, 71, 74), and the consensi differ slightly from study to study. Whether the transcription of a gene is repressed or activated depends on the location of the *cre* relative to the transcriptional start site. When the *cre* is located upstream of the -35 region, binding of the CcpA-HPr- or CcpA-Cpr-complex stimulates transcription (42, 69). Location of the *cre* within the promoter or within the protein coding region on the other hand leads to prevention of transcription initiation (23) or to blockage of elongation (47, 75).

CcpA was also found to bind to *cre* sites in the absence of a corepressor (31-33, 50). Specific inhibition of *in vitro* transcription by CcpA was promoted by the presence of NADP(H). However, this effect was not due to binding of CcpA to the *cre*. (33) Instead, it was proposed that CcpA directly interacts with and inhibits RNA polymerase, a process that is stimulated by NADP(H), independently of the redox state of the cell. (34) In *B. megaterium* the overall NADP(H) concentration was shown to be fairly constant (60), and nothing has been published about the overall NADP(H) concentrations in *S. aureus* in response to the nutritional state of the cell. It is therefore not clear to which signal the interaction of NADP(H) with CcpA corresponds.

In the model organism *B. subtilis*, CcpA was found to regulate about 10% of all genes, most of which encode metabolic enzymes (50). Glycolytic enzymes and genes of the carbon overflow pathways are induced by glucose (36, 62). In contrast, genes required for gluconeogenesis or for the complete oxidation of glucose, such as genes of the tricarboxylic acid (TCA) cycle are repressed (10). CcpA also regulates pyruvate metabolism. This metabolite plays an important role in central metabolism, being involved in several metabolic pathways (64). But the role of CcpA goes far beyond the regulation of carbon metabolism, as

it also plays a role in nitrogen metabolism. For example, in the presence of glucose, CcpA represses several genes which are necessary for the conversion of amino acids into alternative carbon sources. CcpA is also involved in the regulation of 2-oxoglutarate, which is a central link for carbon and nitrogen metabolism (64).

In addition to its role in carbon and nitrogen metabolism, CcpA was reported to regulate the expression of several virulence factors and to be involved in antibiotic resistance. Thus, CcpA was found to be necessary for invasive infection (61) and to influence the regulation of genes for adhesion, invasion and immune evasion in group A *Streptococcus* (2). In *S. pneumoniae,* it had an influence on virulence (27), as well as in *S. mutans* where it additionally was essential for biofilm formation (1, 72). In *S. gordonii*, inactivation of CcpA lead to reduced penicillin tolerance *in vitro* and *in vivo* (9), and in *S. aureus*, CcpA was shown to influence methicillin resistance (16).

We recently described the role of CcpA in virulence, antibiotic resistance (58) and in biofilm formation (57) in *S. aureus*. The aim of this study was to define the CcpA regulon of this pathogen by transcriptome analysis, mainly focussing on metabolic genes. Similar experiments have been carried out for other bacteria, such as *B. subtilis* (41, 50, 73) and *L. lactis* subsp. *cremoris* (76). However, the common characteristic of these analyses was that they were carried out in unbuffered media, leading to changes in pH which could be responsible for secondary effects in gene expression. Lulko *et al.* (41) observed the CcpA regulon in *B. subtilis* over time. But in their study, the amount of glucose varied at the different sampling time points, and again, changes in pH were not taken into account. To avoid these problems, we added the glucose during the exponential growth phase to the cells in a buffered medium, and sampled 30 min afterwards. At this time point the pH was not changed and we could exclude any secondary effects caused by pH changes. This approach gave us a genome wide overview of the genes subject to CcpA-control in *S. aureus* during exponential growth.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. aureus* Newman (20) and its isogenic Δ*ccpA* mutant MST14 (58) were grown in LB medium buffered with 50 mM HEPES (pH 7.5) in Erlenmeyer flasks with a culture to flask volume of 1:5 under vigorous agitation at 37°C to an optical density (OD_{600}) of 1.0. One half of the culture was transferred to a new Erlenmeyer flask and glucose was added to a final concentration of 10 mM, while the other half remained without glucose. Samples for microarray analysis were taken at (OD_{600}) of 1.0 (T0) and after 30 minutes (T30). Total RNA was extracted as previously described (53, 54).

Microarray design and manufacturing. The microarray was manufactured by *in situ* synthesis of 10'807 different oligonucleotide probes of 60 nucleotides length (Agilent, Palo Alto,CA, USA), selected as previously described (12). It covers approximately 99% of all ORFs annotated in strains N315 and Mu50 (37), MW2 (4) and COL (22) including their respective plasmids (54). Extensive experimental validation of this array has been described previously, using CGH, mapping of deletion, specific PCR and quantitative RT-PCR (12, 35).

Expression microarrays. DNA-free total RNA was obtained after DNase treatment (Qiagen). The absence of remaining DNA traces was evaluated by quantitative PCR (SDS 7700; Applied Biosystems, Framing-ham, MA) with assays specific for 16s rRNA (53, 54). Batches of 8 µg total *S. aureus* RNA were labelled by Cy-3 or Cy-5 dCTP using the SuperScript II (Invitrogen, Basel, Switzerland) following manufacturer's instructions. Labelled products were then purified onto QiaQuick columns (Qiagen) and mixed with 250 µl Agilent hybridization buffer, and then hybridized at a temperature of 60°C for 17 hours in a dedicated hybridization oven (Robbins Scientific, Sunnyvale, CA, USA). Slides were washed with Agilent proprietary buffers, dried under nitrogen flow, and scanned (Agilent, Palo Alto, CA, USA) using 100% PMT power for both wavelengths.

Microarray analysis. Fluorescence intensities were extracted using the Feature extraction™ software (Agilent, version 8). Local background-subtracted signals were corrected for unequal dye incorporation or unequal load of labelled product. The algorithm consisted of a rank consistency filter and a curve fit using the default LOWESS (locally weighted linear regression) method. Data consisting of two independent biological experiments were analyzed using GeneSpring 7.3 (Agilent). An additional filter was used to exclude irrelevant values. Background noise of each experiment was evaluated by computing the standard deviation of negative control intensities. Features whose intensities were smaller than the standard deviation value of the negative controls in all the measurements were considered as inefficient hybridization and discarded from further analysis (35). Fluorescence values for genes mapped by 2 probes or more were averaged. Statistical significance of differentially

expressed genes was identified by variance analysis (ANOVA) (14, 54), performed using GeneSpring, including the Benjamini and Hochberg false discovery rate correction (5%). A gene was considered to be regulated by glucose and/or CcpA if transcription was induced or repressed at least two fold.

Evaluation of the microarray data. Several classes of effects could be observed. Genes, which showed differences in total transcriptome between wild type and mutant in the absence of glucose at both time points, e.g. OD_{600} 1 (T0) and after 30 min (T30), were considered to be CcpA-dependent, but glucose-independent. When a difference was only observed at one of the two time points or the gene was upregulated at one and downregulated at the other time point, it was assumed to have fluctuating expression patterns and was not considered in this study. Genes with a differential expression upon glucose addition in the wild type but not in the Δ*ccpA* mutant were considered to be strictly CcpA-dependent. Changes occurring in parallel in the wild type and the mutant were considered to be due to glucose, but CcpA-independent. A last group comprised genes, which were found to be affected in their expression in response to glucose in both wild type and mutant, but with differing ratios, or genes, which showed no regulation in the wild type, but regulation in the mutant upon glucose addition. This group of genes was considered to be partially controlled by CcpA but in an undefined way.
For a better interpretation, the organisation of genes in putative operons was deduced from the transcriptional profiles of adjacent genes over time according to previous microarrays (8) and by searching for putative terminator sequences using TransTerm (15).

Northern blot analyses. For Northern blot analysis cells were centrifuged for 2 min at 12,000 x g and cell-sediments snap-frozen in liquid nitrogen. RNA isolation and Northern blotting were performed as described earlier (43). Primer-pairs are shown in Table 1. All Northern blot analyses were performed at least twice on independently isolated RNA samples.

Table 1: Primers used for construction of DIG-labelled DNA probes

Primer	Sequence (5'-3')	Source or reference
aldA+	ACAATCGAAGTGACTAATCC	This study
aldA-	CGTACGATACTCATTGTGTC	This study
arg+	TGATGCAGTAATTCCAATGC	This study
arg-	AATATACCTGAAGAGTCACC	This study
pflAB+	CATCTGACAATGATGATACC	This study
pflAB-	GGAGTATGTTACAGATCACG	This study
rocA+	GTATTGTCTACAGTATTGCC	This study
rocA-	TTAGAAGAAGCAGGATTACC	This study
rocD+	TAACTTAGGTGAATGGTACG	This study
rocD-	TCTAATGCAGCAATTGATGC	This study

Detection of glucose and acetate

The concentrations of glucose and acetate in the supernatants were determined using commercially available kits (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions.

Urease assay

McFarland 0.5-standard cell suspensions were diluted 100-fold in urea medium (13) and incubated in 12-well plates at 37° for 24 hours. In parallel colony forming units (cfu/ml) were determined.

RESULTS AND DISCUSSION

To evaluate the effect of glucose and the impact of CcpA on the metabolism of *S. aureus* growing in a complex medium, we followed the differential transcriptome of exponentially growing cultures of strain Newman and its Δ*ccpA* mutant upon glucose addition. Glucose triggered acidification of the medium was avoided by sufficient buffering, allowing to maintain the pH over the time period followed.

In line with previous findings (58), the wild type grew slightly faster than the Δ*ccpA* mutant, reaching OD_{600} 1 approximately 20 min earlier than the mutant. The addition of glucose clearly increased the growth rate of the wild type, but had only a minor effect on the mutant (Fig. 1). 60 min after the glucose addition, almost all glucose was depleted down to 0.5 mM in the wild type culture. In the Δ*ccpA* mutant, however, glucose consumption was slower, with 3 mM glucose left in the medium even 60 min after the impulse (Fig. 1). The decreased glucose consumption rate in the Δ*ccpA* mutant culture might be explained by a high phosphorylation rate of HPr at Ser-46, similar to the situation found in *B. subtilis*, where the HPr kinase is highly active in the Δ*ccpA* mutant, and all HPr is phosphorylated at Ser-46. In this state, HPr cannot participate in sugar transport, and the PTS-mediated glucose uptake is largely repressed (40).

Despite increased glucose consumption rates in the wild type, acetate production was only slightly enhanced compared to the mutant. A pH of 7.5 was maintained in both strains and under both growth conditions for over 2 h after glucose addition. Thus pH effects could be ruled out.

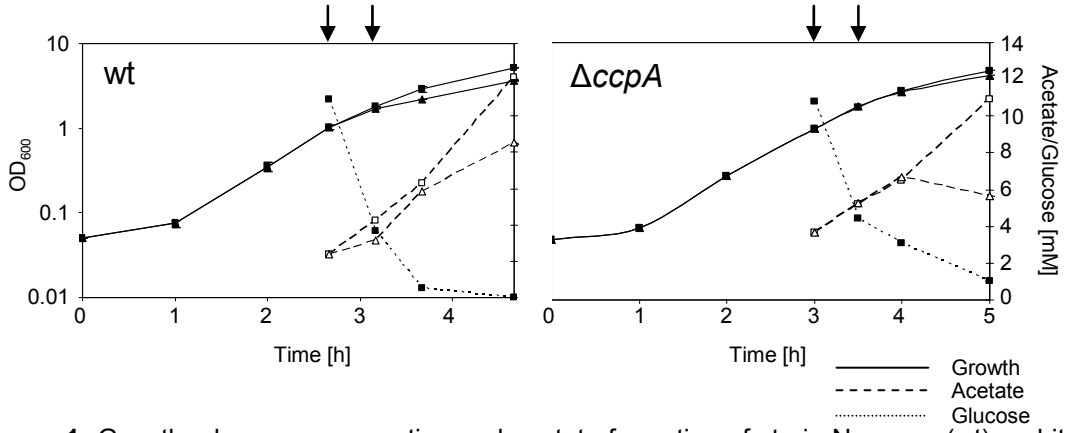

Figure 1: Growth, glucose consumption and acetate formation of strain Newman (wt) and its isogenic Δ*ccpA* mutant (Δ*ccpA*). Cells were grown to OD_{600} 1, cultures were split and 10 mM glucose were added to one half of the culture (squares), while the other half remained without glucose (triangles). Cells were sampled at OD_{600} 1 and 30 min after the impulse for RNA isolation (indicated by arrows).

Differential transcriptome analysis

In all experiments, a gene was considered to be regulated by glucose and/or CcpA if transcription was induced or repressed at least two fold. The overall range of differential gene expression was narrow, peaking around 2-3 fold induction or repression.

The total number of genes, which were expressed at a sufficient level to give meaningfull data was 2479. 111 of these genes had no homologues in strain Newman, and where therefore excluded from the analysis. Of the 2368 remaining genes, a total of 159 was found to be regulated in a CcpA-dependent fashion upon glucose addition. 21 genes seemed to be partially controlled by CcpA, 12 genes exhibited CcpA-independent glucose effects. The largest group comprised 229 genes affected by the Δ*ccpA* mutation in a glucose-independent fashion (Table 2).

Table 2: Numbers of *S. aureus* genes subject to regulation by either glucose or CcpA[a].

Regulatory class[b]	Number of genes
CcpA-dependent, but glucose-independent	**229**
Downregulated	121
Upregulated	108
CcpA- and glucose-dependent	**159**
Downregulated	80
Upregulated	79
Partially dependent on CcpA	**21**
CcpA-independent, but glucose-dependent	**12**
Downregulated	11
Upregulated	1

[a] A gene was considered to be regulated if transcription was induced or repressed at least two fold.
[b] Classes partly overlap.

CcpA-dependent, glucose-independent regulation

Genes with consistent glucose-independent up- or downregulation by CcpA are listed in supplementary Tables 1 and 2.

Of particular interest in this class of genes were the *cap* genes, which are responsible for capsule formation. Although their expression seemed not to be affected by glucose within the time span of 30 min, the wild type showed much higher expression than the Δ*ccpA* mutant. We recently reported that the presence of glucose repressed capsule formation in *S. aureus* in a CcpA-dependent manner (58). However, in that latter study we assessed capsule formation after 24 h growth in the presence or absence of glucose allowing indirect CcpA mediated glucose effects to take place. In addition, transcription of the *cap* operon was shown to be modulated by various other regulators, for example σ^B or the σ^B-dependent *arlRS* and *yabJ-spoVG* loci (45).

Genes of the urease-operon were also upregulated in the wild type compared to the Δ*ccpA* mutant, as confirmed by the increased urease production by the wild type strain in urea-containing medium but not in the Δ*ccpA* mutant (Fig. 2). Urease hydrolyses urea into ammonia and carbon dioxide, supplying nitrogen and helping to maintain the pH stable by the formation of ammonium. Urease is considered to be a virulence factor contributing to pathogenesis in many bacteria (48).

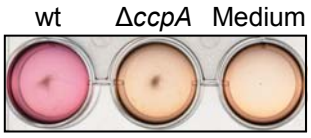

Figure 2: Urease production in urea-containing medium. The increase in pH resulting from the cleavage of urea is indicated by a purple colour. wt, strain Newman; Δ*ccpA,* strain Newman Δ*ccpA.*

CcpA-dependent, but glucose-independent regulation has also been reported in *B. subtilis* and might indicate that CcpA does not stringently need to form a complex with HPr-Ser-P to exert its regulatory function (33, 50). Shelburne et *al*. (61) observed CcpA-DNA interaction in the absence of HPr-Ser-P, however, binding was much stronger in the presence of HPr-Ser-P. Another explanation for this phenomenon was given by Kim et *al.* (33), who searched for potential effectors of CcpA by *in vitro* transcription in *B. subtilis*. They found that CcpA could inhibit transcription in the presence of NADP(H) without DNA binding and suggested that catabolite repression by CcpA could also be a result of direct interactions with the RNA polymerase, a process, that is enhanced by NADP(H). Nothing has been published about the overall NADP(H) concentrations in *S. aureus* cells in response to the nutritional state and further investigations are needed to elucidate the exact mechanism, by which CcpA regulates gene expression in the absence of glucose. More genes of *S. aureus* are subject to glucose-independent CcpA-mediated regulation (229 genes) than to glucose-induced CcpA-mediated regulation (159 genes). This is in contrast to the findings in *B. subtilis,* in which glucose-dependent CcpA-regulated genes by far outweighed genes subject to glucose-independent CcpA-regulation (50), though under different conditions. However, these findings suggest that glucose-independent regulation by CcpA might play a particular important role in *S. aureus*.

Glucose-dependent, CcpA-dependent genes

Supplementary Tables 3 and 4 show all genes which are subject to regulation by glucose in a CcpA-dependent fashion. The tables include a few genes which were not meeting the arbitrary threshold, such as SA0605 or SA0299. These genes were nevertheless listed because they are part of putative operons and showed a tendency towards regulation.

Regulated genes were further analysed by screening for putative *cre*-sites using the *B. subtilis* consensus sequence (WWTGNAARCGNWWWCAWW) suggested by Miwa et *al.* 2000 (47). Being aware that diverse *cre*-site consensi have been published (42, 46, 66, 71,

74), we allowed up to two mismatches in the staphylococcal *cre* candidates, therewith allowing all putative *cre* consensus sequences to be identified. To constrict the *cre*-sites identified, we evaluated the presence of palindromic parts (supplementary Tables 3 and 4). By far not all regulated genes contained a putative *cre* in their vicinity, suggesting that they were not under direct CcpA control. An example of such indirect control is *citB*, which we recently reported to be downregulated by CcpA in the presence of glucose (57). In *B. subtilis*, *citB* is indirectly controlled by CcpA through the regulation of CcpC (30, 31). The presence of a CcpC homologue in *S. aureus* suggests that also in this bacterium *citB* might be regulated by CcpA over CcpC.

For a better global understanding, the regulated genes were grouped into functional categories according to the KEGG database (http://www.genome.jp/kegg/) (29) (Figure 3). The genes belong to 11 different functional categories, with unknown proteins representing the largest regulated category, followed by transport/binding proteins and lipoproteins, metabolism of amino acids, metabolism of carbohydrates, and metabolism of nucleotides and nucleic acids.

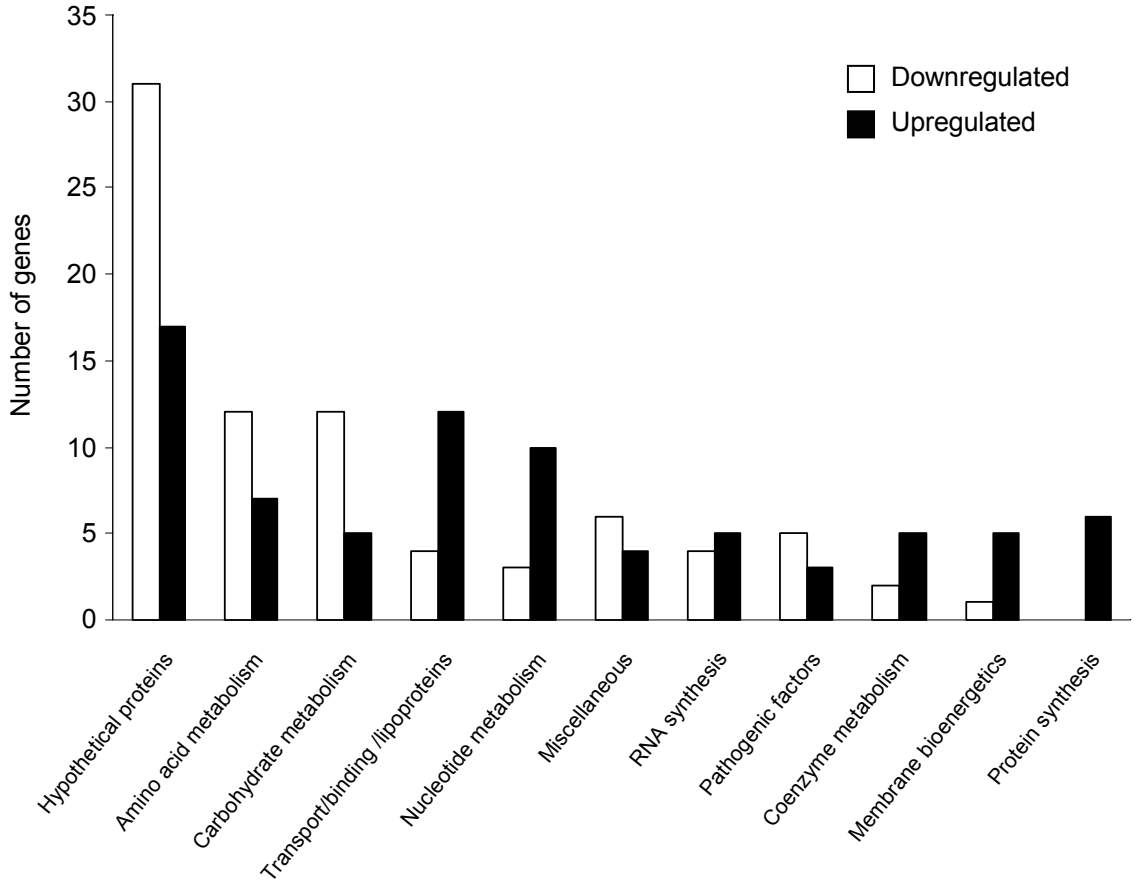

Figure 3: Functional classification and numbers of genes that were found to be regulated by glucose in a CcpA-dependent way. The functional category miscelleneous contains genes of the following categories: adaptation to atypical conditions, DNA recombination, DNA replication, cell division, metabolism of phosphate, sensors, metabolism of lipids.

CcpA-independent regulation by glucose

Still using the threshold of two, we assigned genes that showed similar ratios in glucose induced down- or upregulation in the wild type and in the mutant to a category of genes/operons with CcpA-independent regulation by glucose (Table 3). We found only 12 genes fitting this category, with 11 being downregulated. Two of these genes (*lacA* of the *lac* operon, and SA0321 of the putative SA0318-SA0321 operon), contained a putative, but poorly palindromic *cre*-site in their promoter region.

Table 3: Genes/operons with CcpA-independent regulation by glucose.

ID				wt	mut
N315	Newman	common	Product[a]	+/-[b]	+/-[b]
Downregulated by glucose					
SA0256	NWMN_0200	*bglA*	6-phospho-beta-glucosidase	0.5	0.5
SA0318	NWMN_0322		ascorbate-specific PTS system enzyme IIC	0.1	0.3
SA0319	NWMN_0323		similar to PTS system component	0.2	0.2
SA0320	NWMN_0324		similar to PTS transport system IIA component	0.2	0.2
*SA0321	NWMN_0325		similar to PTS multidomain regulator	0.3	0.2
SA1991	NWMN_2093	*lacG*	6-phospho-beta-galactosidase	0.5	0.5
SA1992	NWMN_2094	*lacE*	PTS system, lactose-specific IIBC component	0.5	0.4
SA1993	NWMN_2095	*lacF*	PTS system, lactose-specific IIA component	0.4	0.4
SA1994	NWMN_2096	*lacD*	tagatose-1,6-diphosphate aldolase	0.5	0.4
SA1995	NWMN_2097	*lacC*	tagatose-6-phosphate kinase	0.6	0.6
SA1996	NWMN_2098	*lacB*	galactose-6-phosphate isomerase LacB subunit	0.5	0.4
*SA1997	NWMN_2099	*lacA*	galactose-6-phosphate isomerase LacA subunit	0.6	0.5
Upregulated by glucose					
SA1842	NWNM_1943	*agrB*	accessory gene regulator B	2.0	2.5
SAS066	NWNM_1944	*agrD*	accessory gene regulator D	1.5	2.1
SA1843	NWNM_1945	*agrC*	accessory gene regulator C	1.3	1.8
SA1844	NWNM_1946	*agrA*	accessory gene regulator A	1.0	1.2

[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website (18) and/or the KEGG website (29).
[b] Comparison of gene expression with (+) and without (-) glucose, genes with a +/- glucose ratio of ≤ 0.5 or ≥2 in the wild-type were considered to be regulated.
* Genes containing *cre*-sites.

Especially interesting in this group is the *lac* operon, which has been described to be under the control of the repressor *lacR* in *S. aureus* (51). *lacR* is located directly upstream of the *lac* operon. However, Oskouian and Stewart (51) proposed the existence of a second regulator acting as catabolite repressor in addition to *lacR*. In *Lactobacillus casei*, CcpA was shown to control the *lac* operon (49). In *S. xylosus*, the *lac*-promoter is under partial control of CcpA, but another, unknown mechanism was postulated to be responsible for catabolite repression

by glucose (5). However, in *S. aureus*, CcpA seems not to be involved in the regulation of the *lac* operon. Also, rapid fluctuations of the *lac* operon expression over growth even in the absence of glucose question the *lac* operon results (data not shown).

Genes under partial control of CcpA

Genes considered to be partially controlled by glucose in a CcpA-dependent manner are shown in Table 4. These genes show miscellaneous regulatory patterns. One pattern comprises parallel up- or downregulation by glucose in wild type and mutant, but with different ratios, as seen in *alsDS* and *treP*. Another set of genes (i.e. *pstB* or *mtlF,* SA1218-1221, SA2321) show divergent glucose-regulation in wild type and mutant. A third set, represented by the *gntPKR* operon, the *ribHABD* operon, SA1961 and SA2434-SA2435 changed expression in response to glucose in the mutant but not in the wild type. *gntPRK* and *ribHABD* contained putative *cre*-sites, 64 and 35 baisepairs upstream of the translational start of the first transcribed gene, respectively, indicating that CcpA regulates their expression by direct binding. In addition to control through CcpA, these genes are probably controlled by other regulatory proteins, as well.

Table 4: Genes under partial control of CcpA[1].

ID				wt	mut
N315	Newman	common	Product[a]	+/-[b]	+/-[b]
SA0432	NWMN_0438	*treP*	PTS system, trehalose-specific IIBC component	0.5	0.2
SA0433	NWNM_0439	*treC*	alpha-phosphotrehalose	0.7	0.3
SA0434	NWNM_0440	*treR*	trehalose operon repressor	0.7	0.3
SA1218	NWNM_1297	*pstB*	phosphate ABC transporter, ATP-binding protein (PstB)	0.5	2.6
SA1219	NWNM_1298		similar to phosphate ABC transporter	0.4	2.7
SA1220	NWNM_1299		similar to phosphate ABC transporter	0.3	3.7
SA1221	NWNM_1300	*pstS*	thioredoxine reductase	0.1	3.6
*SA1586	NWNM_1659	*ribH*	6,7-dimethyl-8-ribityllumazine synthase	0.6	2.2
SA1587	NWNM_1660	*ribA*	riboflavin biosynthesis protein	0.6	1.8
SA1588	NWNM_1661	*ribB*	riboflavin synthase alpha chain	0.7	2.0
SA1589	NWNM_1662	*ribD*	riboflavin specific deaminase	0.7	2.0
SA1960	NWNM_2057	*mtlF*	PTS system, mannitol specific IIBC component	6.4	0.2
SA1961	NWNM_2058		similar to transcription antiterminator BglG family	0.9	0.4
SA2007	NWNM_2110	*alsD*	alpha-acetolactate decarboxylase	9.1	2.7
SA2008	NWNM_2111	*alsS*	alpha-acetolactate synthase	9.1	3.1
SA2293	NWNM_2401	*gntP*	gluconate permease	0.7	2.5
SA2294	NWNM_2402	*gntK*	gluconate kinase	1.6	3.7
*SA2295	NWNM_2403	*gntR*	gluconate operon transcriptional repressor	1.5	3.2

SA2321	NWMN_2432		hypothetical protein	0.1	2.5
SA2434	NWNM_2540		PTS system, fructose-specific IIABC component	1.2	0.4
SA2435	NWNM_2541	*pmi*	mannose-6-phosphate isomerase	1.2	0.4

[1] Genes with parallel glucose-mediated regulation in wild type and mutant but with different ratios, genes with divergent glucose-mediated regulation in wild type and mutant, and genes with glucose-mediated regulation in the mutant but not in the wild type.
[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website and/or the KEGG website.
[b] Comparison of gene expression with (+) and without (-) glucose, genes with a +/- ratio of ≤ 0.5 or ≥2 in the wild-type and the mutant were considered to be regulated).

Metabolic pathways under the control of CcpA

Glycolysis/Gluconeogenesis

In *S. aureus,* glucose is mainly catabolized to pyruvate via glycolysis (11). The enzymes catalyzing the central parts of glycolysis of *S. aureus* are encoded by five genes, coding for glyceraldehyde-3-phosphate dehydrogenase (*gap*), phosphoglycerate kinase (*pgk*), triosephosphate isomerase (*tpi*), phosphoglyceromutase (*pgm*), and enolase (*eno*), and are cotranscribed in an operon. In the presence of glucose, *tpi* and *pgk* were upregulated in a CcpA-dependent manner (Fig. 4, supplementary table 5). The absence of putative *cre*-sites indicates indirect control by CcpA. Almost all genes of the operon showed upregulation by glucose in the wild type but even in the mutant, though below the threshold. This might be due to regulation through additional factors. A possible regulatory mechanism for these genes could involve *gapR*, which is co-transcribed with the glycolytic genes and codes for the glycolytic operon regulator in *S. aureus.* In *B. subtilis*, the product of this gene, also known as CggR (for central glycolytic genes regulator), was found to repress transcription of the *gap* orperon. Fructose-1,6-bisphosphate, which is built in the presence of glucose, inhibits DNA-binding by CggR and thus glycolytic genes are transcribed.

The microarrays confirmed our previous findings (58), in which we found glucose-dependent and CcpA-mediated downregulation of PEP carboxykinase (*pckA*). The presence of a *cre*-site in the promoter region (55, 58) indicates direct regulation by CcpA. This is in contrast to the *B. subtilis pckA,* which was found to be under indirect control of CcpA (10).

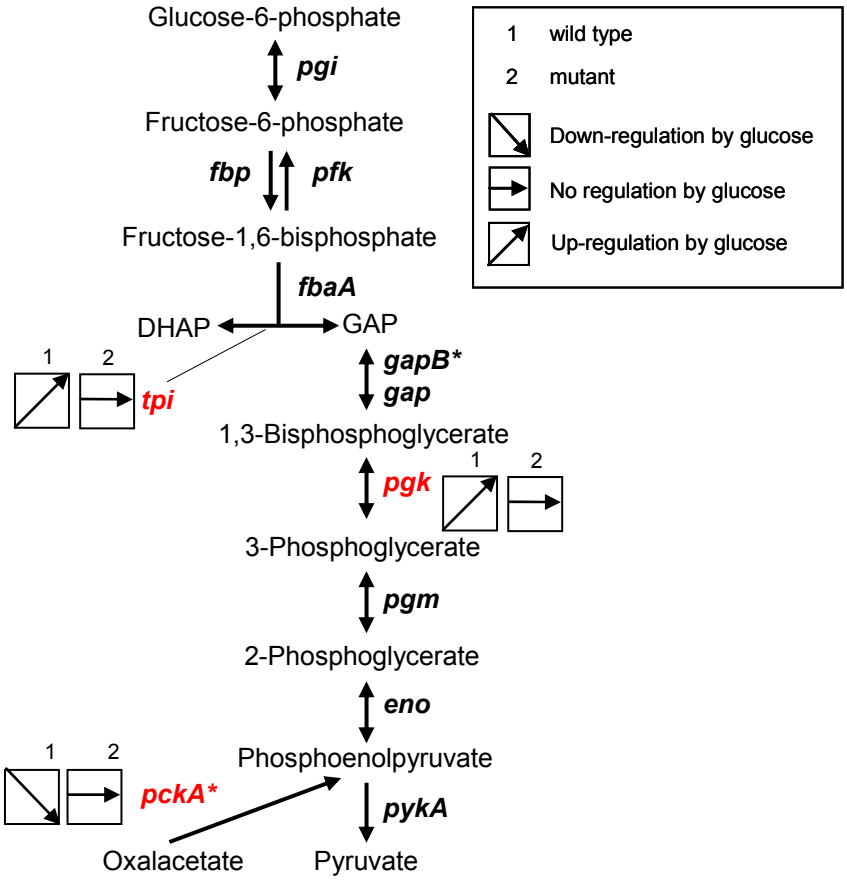

Figure 4: Assignment of genes coding for enzymes of glycolysis and gluconeogenesis which are regulated by CcpA. *eno*, enolase; *fbpA*, fructose-bisphosphate aldolase; *fbp*, fructose-1,6-bisphosphatase; *gap*, glyceraldehyde-3-phosphate dehydrogenase; *gapB*, glyceraldehyde-3-phosphate dehydrogenase; *pckA*, phosphoenolpyruvate carboxykinase; *pfk*, phosphofructokinase; *pgi*, glucose-6-phosphate isomerase; *pgk*, phosphoglycerate kinase; *pgm*, phosphoglycerate mutase; *pykA*, pyruvate kinase; *tpi*, triose-3-phosphate isomerase. *, genes with *cre*-sites; red, regulated genes.

Together, these findings suggest that glycolysis/gluconeogenesis are at least partly controlled by CcpA in *S. aureus* but that most probably other regulatory mechanisms contribute to their regulation as well.

Pentose phosphate pathway

The pentose phosphate pathway is an alternative glucose degradation pathway of *S. aureus* (11). It provides the cell with NADPH and precursors for biomass, which are needed in many anabolic reactions. *gntPKR* was the only operon of this pathway we found to be regulated (Table 3). Surprisingly, these genes showed glucose-mediated upregulation in the mutant, but virtually no regulation in the wild type. These genes may be upregulated by glucose by a CcpA-independent mechanism that might be counteracted by CcpA in the wild type. In *B. subtilis,* genes of the pentose phosphate pathway were not affected by the presence of glucose (10), presenting a difference between *B. subtilis* and *S. aureus* CcpA-mediated regulation.

Carbon overflow

In the presence of high glucose concentrations, many bacteria metabolize a large proportion of the glucose only as far as pyruvate and acetyl-CoA and the TCA cycle is down-regulated. Pyruvate and acetyl-CoA are then further metabolized to by-products of metabolism and excreted into the medium. This mechanism is referred to as carbon overflow and enables the cell to maintain its redox balance and to generate ATP without using the cytochrome system. Products of carbon overflow in *S. aureus* and *B. subtilis* include lactate, acetate and acetoin (36, 58, 62). When glucose is depleted from the medium, the cells reintroduce these by-products into central metabolism.

The genes for acetolactate synthase (*alsS*) and acetolactate decarboxylase (*alsD*), both involved in acetoin production, where upregulated by glucose (Table 3). Although, upregulation was found in wild type and Δ*ccpA* mutant, it was three times higher in the wild type, suggesting that these genes were only partially under the control of CcpA. The absence of a *cre*-site in the promoter region indicated indirect regulation. The *alsSD* (*budBA1*) operon has been shown to be induced under anaerobic conditions (21), indicating that our cultures were growing under microaerophilic conditions.

Although the amount of acetate in the medium increased upon glucose addition in both, wild type and mutant (Fig.1), neither the genes coding for phosphotransacetylase (*pta*) nor for acetate kinase (*ackA*), both enzymes of acetate production, where significantly upregulated by glucose. This is in contrast to the findings in *B. subtilis*, in which not only *alsS*, but also *pta* and *ackA* where found to be induced by glucose in a CcpA-dependent way (26, 52, 62). Genes responsible for acetate and acetoin utilization such as acetyl-CoA synthetase (*acsA*), acetoin dehydrogenase (*acuA*), and the acetoin utilization protein (*acuC*) where found to be repressed by glucose through CcpA in *B. subtilis* (25). This was not the case in *S. aureus*.

Formate acetyltransferase (*pflA*) and its activating enzyme (*pflB*), which are part of pyruvate metabolism, were found to be upregulated by CcpA in our microarrays. These data could not be confirmed by Northern blot analysis, where we failed to find an upregulation in the wild type (Fig. 5). However, as in the microarrays, transcription was much higher in the mutant than in the wild type, in accordance with the findings of Asunama et *al*. (3), who found higher *pfl* transcription in a Δ*ccpA* mutant of *Streptococcus bovis*.

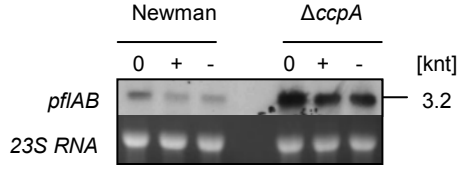

Figure 5: Transcription of *pflAB* in strain Newman and its Δ*ccpA* mutant in response to glucose. Cells were grown to OD_{600} 1, cultures where split and glucose added to one half (+), while the other half remained without glucose (-). RNA was sampled at OD_{600} 1, and after 30 min. Data are representative for two independent experiments.

Carbon overflow and pyruvate metabolism thus represent two other metabolic pathways, which differ in terms of CcpA-mediated regulation between *S. aureus* and *B. subtilis.*

TCA cycle

As in *B. subtilis*, CcpA repressed several genes of the TCA cycle in *S. aureus*, including *citBCZ, sdhB, sucCD* and *odhAB* (Fig. 6, supplementary Table 6). The promoter region of *citZ*, which is co-transcribed with *citB* contains a putative *cre*-site (supplementary Table 1). Also *odhAB* and *sucCD* exhibit putative *cre*-sites, indicating that the TCA cycle is under direct control of CcpA. These findings are very similar to those in *B. subtilis.*

The *pdhABCD* operon, coding for the pyruvate dehydrogenase complex, which links glycolysis to TCA cycle, was not induced by glucose or CcpA in *S. aureus*, in contrast to *B. subtilis*, in which *pdhABCD* was activated by glucose (10).

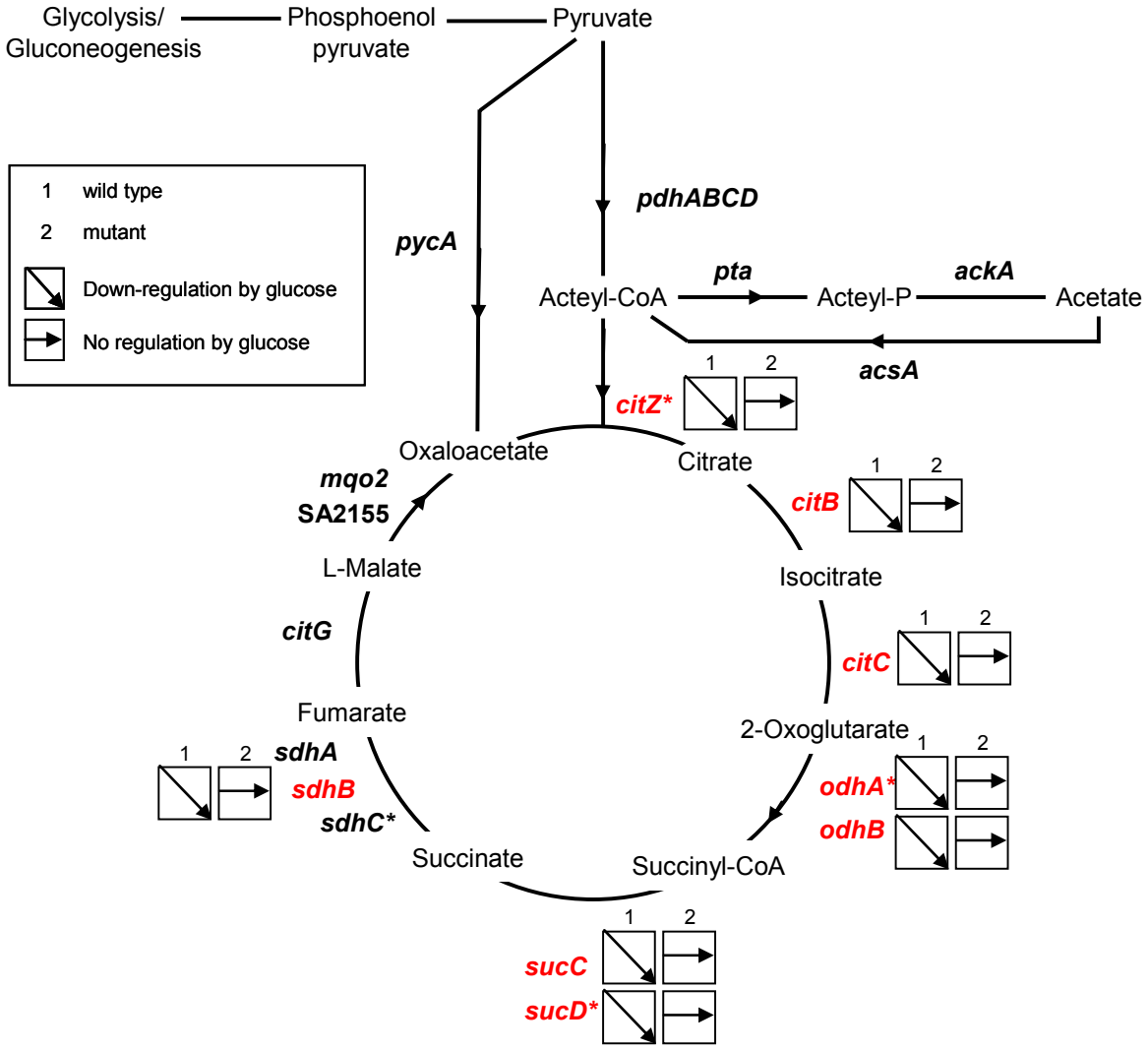

Figure 6: Assignment of genes coding for enzymes the TCA cycle, which are regulated by CcpA. The genes code for the following enzymes: *ackA*, acetate kinase; *acsA*, acetyl-CoA synthetase; *citB*, aconitate hydratase; *citC,* citrate dehydrogenase; *citG*, fumarate hydratase; *citZ,* citrate synthase; *mqo2*, malate:quinone-oxidoreductase; *odhA*, 2-oxoglutarate dehydrogenase component E1; *odhB,* 2-oxoglutarate dehydrogenase component E2; *pckA*, phosphoenolpyruvate carboxykinase; *pdhABCD*, pyruvate dehydrogenase *pycA*, pyruvate carboxylase; SA2155, malate:quinone-oxidoreductase; *sdhA*, succinate dehydrogenase; *sucC*, succinyl-CoA synthetase, beta subunit; *sucD*, succinyl-CoA synthetase, alpha subunit. *, genes with *cre*-sites; red, regulated genes.

Amino acid degradation

S. aureus is able to use amino acids as secondary carbon sources. However, this is not necessary in the presence of high amounts of glucose. Accordingly, we found that many genes coding for enzymes of amino acid degradation were repressed by glucose in a CcpA-dependent fashion (Fig. 7, supplementary table 7).

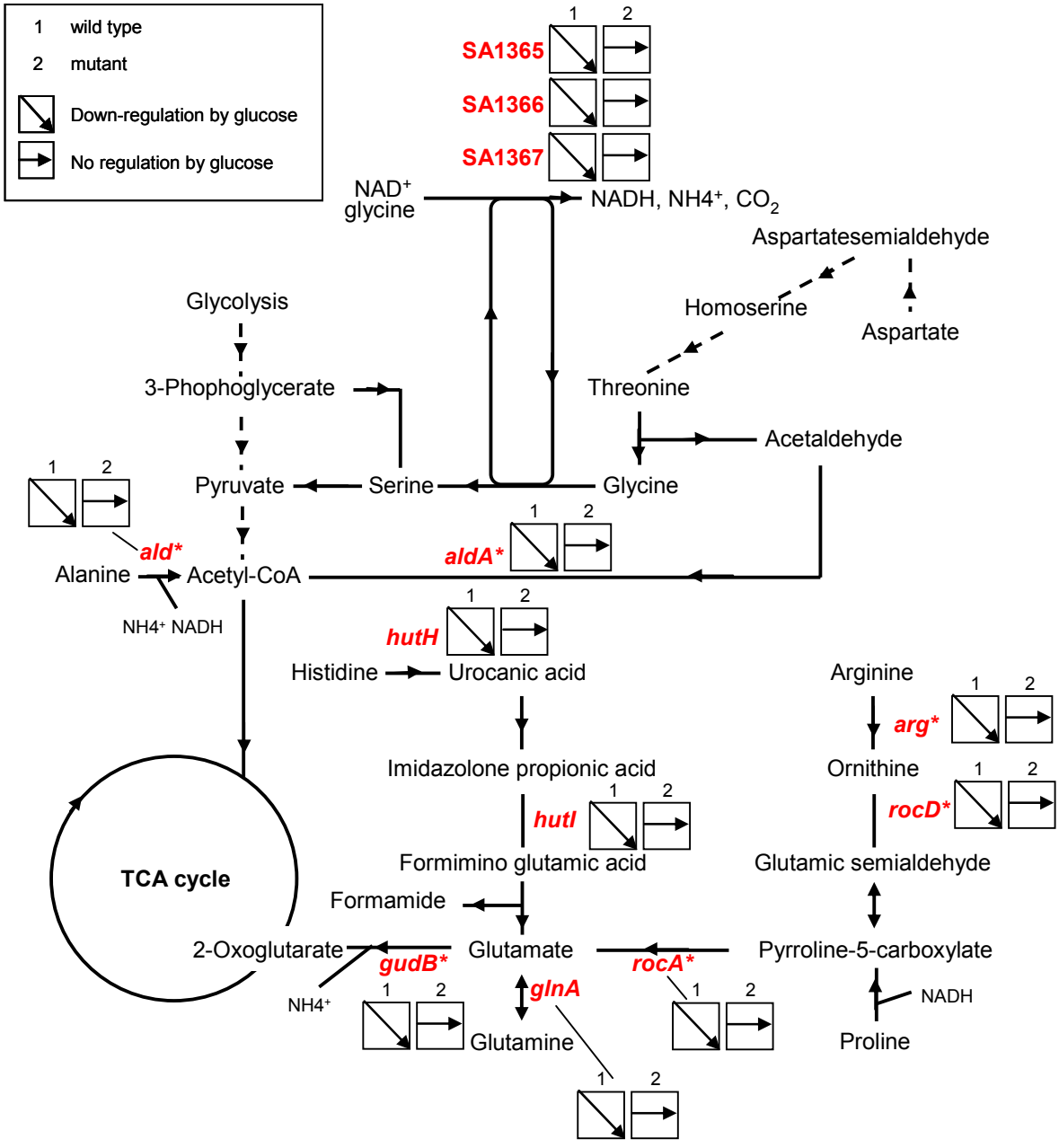

Figure 7: Assignment of genes coding for enzymes of amino acid degradation, which are regulated by CcpA. The genes code for the following enzymes: *ald*, alanine dehydrogenase; *aldA*, aldehyde dehydrogenase; *arg*, arginase; *glnA*, glutamine synthetase; *gudB*, glutamate dehydrogenase; *hutH*, histidine ammonia-lyase *hutI*, imidazolonepropionase; *rocA*, delta-1-pyrroline-5-carboxylate dehydrogenase; *rocD*, ornithine aminotransferase; SA1334, pyrroline-5-carboxylate reductase; SA1365, glycine dehydrogenase subunit 2; SA1366, glycine dehydrogenase subunit 1; SA1367, aminomethyltransferase. Only regulated genes are shown. *, genes with *cre*-sites.

Many of these genes (*aldA, rocD, gudB, ald, arg,* and *rocA*) contain a putative *cre*-site in their promoter region (supplementary Table 3) and might therefore be under the direct control of CcpA. Northern blot analyses, which were performed with some of these genes, confirmed the findings of the microarray (Fig. 8). Degradation of amino acids yields citric acid cycle intermediates or their precursors that can be further metabolized and used in gluconeogenesis. For example, arginine, glutamine, and proline can be converted into glutamate and subsequently into 2-oxoglutarate, which is consequently an important link between carbon and nitrogen metabolism (64). Glutamate dehydrogenase (*gudB*) catalyses the reaction from glutamate to 2-oxoglutarate is glutamate dehydrogenase. According to our Northern blots and according to previously published microarray data (8), *gudB* is co-transcribed with *rocD*, coding for ornithine aminotransferase. The operon contains three *cre*-sites (supplementary Table 3). In both, our Northern blots and the microarray, *gudB* and *rocD* genes were down-regulated by CcpA in the presence of glucose.

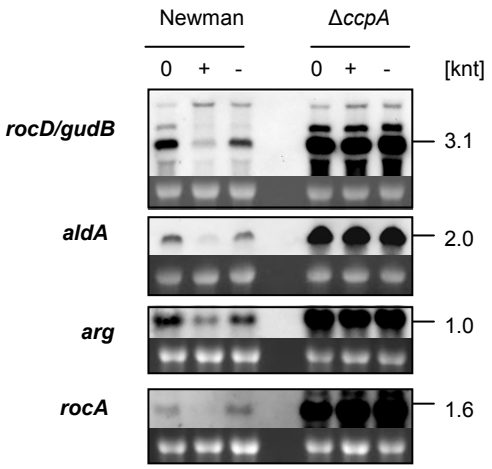

Figure 8: Transcription of *rocD/gudB, aldA, arg,* and *rocA* in strain Newman and its Δ*ccpA* mutant in response to glucose. Cells were grown to OD_{600} 1, cultures where split and glucose added to one half (+), while the other half remained without glucose (-). RNA was sampled at OD_{600} 1, and after 30 min. Data are representative for at least two independent experiments.

The enzyme, which catalyses the inverse reaction, i.e. the conversion of glutamate to 2-oxoglutarate, is catalyzed by glutamate synthase (*gltBD*). We found no CcpA-dependent regulation of these genes, which is in contrast to the findings in *B. subtilis*, in which the transcription of these genes is induced by glucose and requires CcpA (64). This is another aspect in which *S. aureu*s and *B. subtilis* differ in terms of CcpA-regulation. Nevertheless, our findings underline the importance of CcpA in regulating amino acid degradation and in linking the central carbon with the amino acid metabolism.

Branched-chain amino acid synthesis

A key element that balances the distribution of pyruvate and 2-oxoglutarate is the pathway for the biosynthesis of branched-chain amino acids (BCAAs) (64). However, none of the genes devoted to BCAA biosynthesis where found to be induced by the presence of glucose in a CcpA dependent manner in *S. aureus*, which is in contrast to the findings in *B. subtilis*

(40, 63, 68). However, in a transcriptome analysis over time, Lulko et *al*. (41) only observed CcpA-mediated regulation of these genes in the late-exponential growth (transition) phase in *B. subtilis*. Thus, it is possible, that also in *S. aureus* these genes might be regulated by glucose in a CcpA-dependent manner at a later time point.

Transporters and lipoproteins

We found 29 genes coding for transporters or lipoproteins to be regulated by glucose in a CcpA-dependent manner or to be at least partially controlled by CcpA (Table 5). Seven of these genes contained putative *cre*-sites in their promoter region, or as in the case of SA0186, SA0302, and *gntP*, belonged to an operon which contained a putative *cre*-site and were probably under the direct control of CcpA. The upregulation of the glucose uptake protein homolog (SA2053) might be another reason for the rapid glucose consumption observed in the wild type (Fig. 1). Many non-sugar-transporters were found to be regulated by CcpA, such as the *opu*-operon, coding for an osmoprotectant transport system, or for transporters for ammonium, pyrimidine nucleosides or amino acids. These findings reveal that the role of CcpA goes beyond the regulation of central carbon metabolism.

Table 5: CcpA-dependent genes coding for transport/binding proteins and lipoproteins regulated by glucose

ID				wt	mut
N315	Newman	common	Product[a]	+/-[b]	+/-[b]
Downregulated by glucose					
SA0100	NWMN_0049		similar to Na+ P_i-cotransporter	0.2	1.7
*SA0186	NWNM_0136		sucrose-specific PTS tranporter IIBC component protein	0.4	1.2
*SA0302	NWNM_0255		probable pyrimidine nucleoside transport protein	0.4	1.8
*SA1848	NWNM_1950	*nrgA*	probable ammonium transporter	0.4	0.8
SA2226	NWNM_2337		similar to D-serine/D-alanine/glycine transporter	0.2	0.9
SA2227	NWNM_2337		amino acid ABC transporter homolog	0.1	0.9
Upregulated by glucose					
SA0166	NWNM_0116		similar to nitrate transporter	2.8	1.1
SA0167	NWNM_0117		similar to membrane lipoprotein SrpL	2.8	1.6
SA0168	NWNM_0118		similar to probable permease of ABC transporter	2.3	1.1
*SA0214	NWMN_0158	*uhpT*	hexose phosphate transport protein	2.1	1.1
SA0334	NWMN_0339		sec-independent protein translocase protein TatC	2.3	1.1
SA0335	NWMN_0340		twin-arginine translocation protein TatA	2.2	1.4
SA0374	NWNM_0379	*pbuX*	xanthine permease	7.2	1.1

*SA0655	NWNM_0669	*fruA*	fructose specific permease	2.4	1.3
SA0769	NWNM_0780		D-methionine transport system ATP-binding protein	2.4	0.8
SA0770	NWNM_0781		D-methionine transport system permease	2.4	1.0
SA1270	NWNM_1347		similar to amino acid permease	2.0	1.1
SA2053	NWNM_2158		glucose uptake protein homolog	2.5	1.2
SA2234	NWMN_2344	*opuCD*	probable glycine betaine/carnitine/choline ABC transporter (membrane part) OpuCD	1.6	1.2
SA2235	NWMN_2345	*opuCC*	glycine betaine/carnitine/choline ABC transporter (osmoprotection) OpuCC	1.9	1.2
SA2236	NWMN_2346	*opuCB*	probable glycine betaine/carnitine/choline ABC transporter (membrane part) OpuCB	1.9	1.1
*SA2237	NWMN_2347	*opuCA*	glycine betaine/carnitine/choline ABC transporter (ATP-binding) OpuCA	2.6	1.0
SA2239	NWNM_2349		similar to amino acid transporter	2.2	1.1
SA2443	NWMN_2549		similar to accessory secretory protein Asp3	2.0	1.2
SA2444	NWMN_2550		similar to accessory secretory protein Asp2	2.3	1.3
Partially controlled by CcpA					
SA0432	NWMN_0438	*treP*	PTS system, trehalose-specific IIBC component	0.5	0.2
SA1218	NWNM_1297	*pstB*	phosphate ABC transporter, ATP-binding protein (PstB)	0.5	2.6
SA1219	NWNM_1298		similar to phosphate ABC transporter	0.4	2.7
SA1220	NWNM_1299		similar to phosphate ABC transporter	0.3	3.7
SA1960	NWNM_2057	*mtlF*	PTS system, mannitol specific IIBC component	6.4	0.2
*SA2293	NWNM_2401	*gntP*	gluconate permease	0.7	2.5
SA2434	NWNM_2540		PTS system, fructose-specific IIABC component	1.2	0.4

[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website (18) and/or the KEGG website (29).
[b] Comparison of gene expression with (+) and without (-) glucose, genes with a +/- glucose ratio of ≤ 0.5 or ≥2 in the wild-type were considered to be regulated.
*Genes containing *cre*-sites.

Pathogenicity factors

The microarray confirmed our previous findings, in which we observed CcpA-dependent downregulation of *spa,* coding for protein A (Table 6) (58). Also genes coding for other antigens, namely the immunodominant antigen A (IsaA), the staphylococcal secretory antigen SsaA and a homologue of SsaA, where downregulated by glucose in the wild type. SsaA and IsaA are both known to be under the control of WalKR (19), however, this two-

component system was not controlled by CcpA. *Atl* gene, coding for the bifunctional autolysin, was downregulated by glucose in the wild type strain. This is partially in contrast to previous findings, in which we observed a trend towards stronger *atl* expression in glucose containing BHI medium in the wild type in comparison to a Δ*ccpA* mutant (57). However, growth conditions and strains differed between the two studies.

The genes coding for the two-component-system VraSR were found to be upregulated by glucose in a CcpA-dependent manner. This system was reported to regulate the genes of the so-called cell wall stress stimulon, a set of genes that is induced in the presence of cell wall damaging agents (7). Indeed some of the genes, which were reported to belong to the cell wall stress stimulon of strain Newman (44), were found to be also regulated by glucose in a CcpA-dependent manner. However, there was no specific correlation between up- and downregulation in response to glucose and vancomycin.

Although *rsbW*, coding for the anti-sigma B factor was upregulated twofold by glucose in the wild type in a CcpA-dependent manner, none of the other cotranscribed genes of the sigma B operon, i.e. *rsbVU* and σ^B (59) showed increased expression (Table 4). Also the transcription of *asp23,* a model indicator of σ^B activity, showed no CcpA-dependent regulation above the threshold under these conditions, indicating CcpA did neither influence σ^B transcription nor activity.

Surprisingly, we found *rbf*, a gene involved in glucose and salt dependent biofilm formation (38), to be upregulated by glucose in a CcpA-dependent manner. We previously described the role of CcpA in biofilm formation (57) and did not find *rbf* regulation by CcpA under the conditions used in that study. However, the microarray data suggest, that CcpA-dependent regulation of *rbf* might play a role in biofilm formation only under specific conditions.

The gene coding for the fibronectin binding protein B (*fnbB*), was upregulated in the wild type by glucose. Although this protein is truncated and not functional in strain Newman (24), it might be regulated by CcpA in strains where it is functional, suggesting, that CcpA may affect also adherence and host cell invasion.

The microarray confirmed previously published data, that *cidA* transcription is higher in the wild type than in the Δ*ccpA* mutant (57). CidA, controlling cell lysis and the release of eDNA, was shown to contribute to biofilm formation, which is strongly induced in the presence of glucose (57).

Surprisingly, *agrB* of the *agr* locus was found to be upregulated by glucose in wild type and mutant. However, none of the other *agr*-genes which are cotranscribed with *agrB* showed upregulation above threshold level.

Table 6: Regulators and factors involved in virulence and/or resistance and are subject to regulation by CcpA and/or glucose

ID				wt	mut
N315	Newman	common	Product[a]	+/-[b]	+/-[b]
CcpA-dependent regulation					
Downregulated by glucose					
*SA0107	NWNM_0055	*spa*	immunoglobulin G binding protein A precursor	0.2	1.1
SA0620	NWNM_0634		secretory antigen SsaA homologue	0.4	0.9
SA0841	NWNM_0851		similar to cell surface protein Map-w	0.4	0.9
SA0905	NWNM_0922	*atl*	autolysin (N-acetylmuramyl-L-alanine amidase and endo-b-N-acetylglucosaminidase)	0.4	1.1
SA2353	NWNM_2466		similar to secretory antigen precursor SsaA	0.5	1.0
SA2356	NWNM_2469	*isaA*	immunodominant antigen A	0.4	0.8
Upregulated by glucose					
SA0622	NWNM_0636	*rbf*	similar to AraC/XylS family transcriptional regulator	2.2	1.0
SA1009	NWNM_1075		similar to exotoxin 1	2.2	0.8
SA1010	NWNM_1076		similar to exotoxin 4	2.3	0.6
SA1700	NWNM_1822	*vraR*	two-component response regulator	2.2	0.8
SA1701	NWNM_1823	*vraS*	two-component sensor histidine kinase	2.5	0.7
SA1869	NWNM_1970	*sigB*	sigma factor B	1.7	1.0
SA1870	NWNM_1971	*rsbW*	anti-sigmaB factor	2.2	1.1
SA1871	NWNM_1972	*rsbV*	anti-sigmaB factor antagonist	1.3	0.9
SA1872	NWNM_1973	*rsbU*	sigmaB regulation protein RsbU	0.9	0.7
SA2290	NWNM_2397	*fnbB*	fibronectin-binding protein homolog	2.6	1.5
*SA2329	NWNM_2440	*cidA*	murein hydrolase regulator	3.5	1.4
CcpA-independent regulation					
SA1842	NWNM_1943	*agrB*	accessory gene regulator B	2.0	2.5
SAS066	NWNM_1944	*agrD*	accessory gene regulator D	1.5	2.1
SA1843	NWNM_1945	*agrC*	accessory gene regulator C	1.3	1.8
SA1844	NWNM_1946	*agrA*	accessory gene regulator A	1.0	1.2

[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website (18) and/or the KEGG website (29).
[b] Comparison of gene expression with (+) and without (-) glucose, genes with a +/- glucose ratio of ≤ 0.5 or ≥2 in the wild-type were considered to be regulated.
* Genes containing *cre*-sites.

Many virulence-associated genes, such as α-hemolysin or other excreted proteins are expressed in later stages than exponential growth. To observe their regulation and CcpA dependence, further analysis at a later time points would be necessary.

CONCLUSION

This study identified the CcpA regulon of exponentially growing cells in *S. aureus* for the first time. As in other bacteria, the CcpA-regulon of *S. aureus* comprised a large amount of metabolic genes but also genes associated with virulence. Although overall regulation of central carbon metabolism mediated by CcpA was found to be similar to the one in the model organism *B. subtilis,* the extent to which this control was exerted seemed to differ in some aspects between the two bacteria. In general, overall induction or repression levels of CcpA were low, showing mostly values around the threshold level of 2 and 0.5, respectively. However, inactivation of CcpA still leads to changes in metabolism and has an influence on resistance, virulence and biofilm formation (57, 58). One interesting outcome of this study concerns the genes that were regulated by CcpA even in the absence of glucose. This group of genes comprised even more genes than the group with glucose-dependent, CcpA-dependent genes. This finding suggests that glucose-independent regulation by CcpA might be of particular importance in *S. aureus* and needs further investigation.

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Supplementary Table 1: Genes with higher expression in mutant.

ID N315	Newman	common	Product[a]	wt/mut T0[b]	wt/mut T30[b]
SA0107	NWMN_0055	*spa*	immunoglobulin G binding protein A precursor	0.4	0.2
SA0108	NWMN_0056	*sarH1*	staphylococcal accessory regulator A homologue	0.4	0.2
SA0128	NWMN_0077	*sodM*	superoxide dismutase	0.6	0.5
SA0131	NWMN_0080	*pnp*	purine nucleoside phosphorylase	0.6	0.2
SA0143	NWMN_0094	*adhE*	alcohol-acetaldehyde dehydrogenase	0.3	0.3
SA0183	NWMN_0133	*glcA*	PTS enzyme II (EC 2.7.1.69), glucose-specific, factor IIA homologue	0.4	0.2
SA0213	NWMN_0157		conserved hypothetical protein	0.4	0.3
SA0216	NWMN_0160		similar to two-component sensor histidine kinase	0.5	0.4
SA0218	NWMN_0162	*pflB*	formate acetyltransferase	0.3	0.2
SA0219	NWMN_0163	*pflA*	formate acetyltransferase activating enzyme	0.3	0.2
SA0231	NWMN_0175		similar to flavohemoprotein	0.5	0.7
SA0299	NWMN_0253		similar to carbohydrate kinase, PfkB family	0.3	0.1
SA0300	NWMN_0253		truncated hypothetical protein	0.1	0.1
SA0301	NWMN_0254		conserved hypothetical protein	0.1	0.1
SA0302	NWMN_0255		probable pyrimidine nucleoside transport protein	0.2	0.1
SA0304	NWMN_0257	*nanA*	N-acetylneuraminate lyase subunit	0.7	0.3
SA0305	NWMN_0258		similar to glucokinase	0.6	0.4
SA0354	NWMN_0359	*rpsR*	30S ribosomal protein S18	0.7	0.5
SA0374	NWMN_0379	*pbuX*	xanthine permease	0.6	0.4
SA0376	NWMN_0381	*guaA*	GMP synthase (glutamine-hydrolyzing)	0.7	0.5
SA0387	NWMN_0394	*set11*	exotoxin 11 (Pathogenicity island SaPIn2)	0.2	0.2
SA0389	NWMN_0396	*set13*	exotoxin 13 (Pathogenicity island SaPIn2)	0.7	0.5
SA0390	NWMN_0397	*set14*	exotoxin 14 (Pathogenicity island SaPIn2)	0.5	0.3
SA0433	NWMN_0439		alpha-glucosidase	0.5	0.2
SA0434	NWMN_0440		similar to trehalose operon transcriptional repressor	0.6	0.3
SA0452	NWMN_0457	*veg*	VEG protein homologue	0.4	0.4
SA0479	NWMN_0483	*nupC*	pyrimidine nucleoside transport protein	0.7	0.5
SA0495	NWMN_0499	*rplK*	50S ribosomal protein L11	0.5	0.5
SA0496	NWMN_0500	*rplA*	50S ribosomal protein L1 (BL1)	0.6	0.3
SA0497	NWMN_0501	*rplJ*	50S ribosomal protein L10 (BL5)	0.7	0.5
SA0562	NWMN_0577	*adh1*	alcohol dehydrogenase I	0.3	0.4
SA0576	NWMN_0591		hypothetical protein	0.5	0.4
SA0598	NWMN_0612	*pbp4*	penicillin binding protein 4	0.4	0.6
SA0642	NWMN_0656		similar to cobalamin synthesis related protein	0.7	0.5
SA0687	NWMN_0701	*nrdF*	ribonucleoside-diphosphate reductase minor subunit	0.5	0.7
SA0769	NWMN_0780		D-methionine transport system ATP-binding protein	0.6	0.4
SA0770	NWMN_0781		D-methionine transport system permease	0.6	0.4
SA0820	NWMN_0830	*glpQ*	glycerophosphoryl diester phosphodiesterase	0.7	0.5
SA0868	NWMN_0880	*nrdE*	ribonucleoside-diphosphate reductase major subunit	0.6	0.4
SA0908	NWMN_0925		conserved hypothetical protein	0.6	0.5
SA0923	NWMN_0939	*purM*	phosphoribosylformylglycinamidine cyclo-ligase PurM	0.6	0.5
SA0924	NWMN_0940	*purN*	phosphoribosylglycinamide formyltransferase	0.5	0.4
SA0925	NWMN_0941	*purH*	bifunctional purine biosynthesis protein	0.4	0.3

			PurH		
SA0926	NWMN_0942	*purD*	phosphoribosylamine--glycine ligase PurD	0.7	0.5
SA0937	NWMN_0952		cytochrome D ubiquinol oxidase subunit 1 homolog	0.4	0.6
SA0938	NWMN_0953		cytochrome D ubiquinol oxidase subunit II homolog	0.3	0.5
SA1000	NWMN_1066		similar to fibrinogen-binding protein	0.4	0.7
SA1001	NWMN_1067		hypothetical protein	0.4	0.3
SA1009	NWMN_1075		similar to exotoxin 1	0.5	0.2
SA1010	NWMN_1076		similar to exotoxin 4	0.3	0.1
SA1011	NWMN_1077		similar to exotoxin 3	0.5	0.3
SA1012	NWMN_1078	*argF*	ornithine carbamoyltransferase	0.4	0.3
SA1013	NWMN_1079		hypothetical protein, similar to carbamate kinase	0.3	0.3
SA1081	NWMN_1148	*rpsP*	30S ribosomal protein S16	0.5	0.4
SA1099	NWMN_1166	*rpsB*	30S ribosomal protein S2	0.7	0.4
SA1162	NWMN_1238		hypothetical protein	0.6	0.3
SA1240	NWMN_1321		conserved hypothetical protein	0.6	0.5
SA1241	NWMN_1322		similar to nitric-oxide reductase	0.6	0.5
SA1269	NWMN_1346		Blt-like protein	0.4	0.4
SA1270	NWMN_1347		similar to amino acid permease	0.2	0.3
SA1271	NWMN_1348		threonine deaminase IlvA homolog	0.2	0.3
SA1272	NWMN_1349		alanine dehydrogenase	0.1	0.2
SA1301	NWMN_1378	*ndk*	nucleoside diphosphate kinase	0.6	0.4
SA1442	NWMN_1515		similar to caffeoyl-CoA O-methyltransferase	0.4	0.4
SA1472	NWMN_1548		conserved hypothetical protein	0.6	0.5
SA1473	NWMN_1549	*rplU*	50S ribosomal protein L21 (BL20)	0.6	0.5
SA1493	NWMN_1563	*hemD*	uroporphyrinogen III synthase	0.5	0.4
SA1494	NWMN_1564	*hemC*	porphobilinogen deaminase	0.5	0.4
SA1495	NWMN_1565	*hemX*	HemA concentration negative effector *hemX*	0.6	0.5
SA1497	NWMN_1567		conserved hypothetical protein	0.5	0.5
SA1499	NWMN_1569	*tig*	trigger factor (prolyl isomerase)	0.5	0.5
SA1503	NWMN_1573	*rpmI*	50S ribosomal protein L35	0.3	0.4
SA1504	NWMN_1574	*infC*	translation initiation factor IF-3 infC	0.3	0.4
SA1506	NWMN_1576	*thrS*	threonyl-tRNA synthetase 1	0.2	0.3
SA1533	NWMN_1605	*ackA*	acetate kinase homolog	0.4	0.4
SA1554	NWMN_1626	*acsA*	acetyl-CoA synthetase	0.5	0.2
SA1555	NWMN_1627	*acuA*	acetoin dehydrogenase homolog	0.5	0.3
SA1569	NWMN_1641		conserved hypothetical protein	0.5	0.6
SA1585	NWMN_1658		proline dehydrohenase homolog	0.5	0.2
SA1608	NWMN_1680	*metK*	S-adenosylmethionine synthetase	0.5	0.3
SA1617	NWMN_1688		hypothetical protein	0.7	0.5
SA1649	NWMN_1722		conserved hypothetical protein	0.5	0.7
SA1701	NWMN_1823	*vraS*	two-component sensor histidine kinase	0.7	0.3
SA1702	NWMN_1824		conserved hypothetical protein	0.7	0.4
SA1710	NWMN_1832		hypothetical protein, similar to DNA polymerase III, alpha chain PolC type	0.4	0.5
SA1755	NWMN_1877		hypothetical protein	0.4	0.4
SA1813	NWMN_1928		similar to leukocidin chain *lukM* precursor	0.5	0.7
SA1847	NWMN_1949	*scrR*	sucrose operon repressor	0.7	0.3
SA1849	NWMN_1951		conserved hypothetical protein	0.5	0.4
SA1929	NWMN_2031	*pyrG*	CTP synthase	0.4	0.4
SA1960	NWMN_2057	*mtlF*	PTS system, mannitol specific IIBC component	0.6	0.3
SA2007	NWMN_2110	*alsD*	similar to alpha-acetolactate decarboxylase	0.2	0.5
SA2008	NWMN_2111	*alsS*	alpha-acetolactate synthase	0.2	0.6
SA2022	NWMN_2125	*rplQ*	50S ribosomal protein L17 (BL15) (BL21)	0.7	0.3
SA2023	NWMN_2126	*rpoA*	DNA-directed RNA polymerase alpha	0.7	0.3

			chain		
SA2163	NWMN_2275		hypothetical protein	0.5	0.5
SA2168	NWMN_2280		hypothetical protein	0.6	0.5
SA2171	NWMN_2283		hypothetical protein	0.5	0.4
SA2172	NWMN_2284	*gltT*	proton/sodium-glutamate symport protein	0.6	0.5
SA2173	NWMN_2285		hypothetical protein	0.3	0.4
SA2187	NWMN_2299	*nirD*	assimilatory nitrite reductase	0.5	0.3
SA2188	NWMN_2300	*nirB*	nitrite reductase	0.5	0.4
SA2192	NWMN_2304		hypothetical protein	0.4	0.4
SA2201	NWMN_2312		similar to ABC transporter, permease protein	0.5	0.5
SA2202	NWMN_2313		similar to ABC transporter, periplasmic amino acid-binding protein	0.5	0.5
SA2206	NWMN_2317	*sbi*	IgG-binding protein SBI	0.4	0.5
SA2302	NWMN_2412		similar to ABC transporter	0.3	0.2
SA2303	NWMN_2413		similar to membrane spanning protein	0.5	0.3
SA2304	NWMN_2414	*fbp*	fructose-bisphosphatase	0.6	0.3
SA2410	NWMN_2515	*nrdD*	anaerobic ribonucleoside-triphosphate reductase	0.3	0.7
SA2423	NWMN_2529	*clfB*	clumping factor B	0.3	0.2
SA2425	NWMN_2531	*arcC*	carbamate kinase	0.4	0.6
SA2432	NWMN_2538		conserved hypothetical protein	0.4	0.4
SA2433	NWMN_2539		similar to transcription antiterminator BglG family	0.4	0.2
SA2434	NWMN_2540		fructose phosphotransferase system enzyme *fruA* homolog	0.2	0.1
SA2435	NWMN_2541	*pmi*	mannose-6-phosphate isomerase	0.1	0.1
SA2480	NWMN_2586	*drp35*	Drp35	0.7	0.1
SA2496	NWMN_2607		hypothetical protein	0.4	0.4
SAS052	NWMN_1613	*rpsD*	30S ribosomal protein S4	0.6	0.5
SAS091	NWMN_2600		hypothetical protein	0.6	0.1
SAS093	NWMN_2614	*rpmH*	50S ribosomal protein L34	0.5	0.6

[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website (18) and/or the KEGG website (29).
[b] Comparison of gene expression of wild type (wt) and ΔccpA mutant (mut) at OD_{600} 1 (T0) and 30 min later (T30). Genes with a wt/mut ratio of ≤ 0.5 or ≥2 were considered to be regulated.

Supplementary Table 2: Genes with higher expression in wild type.

ID				wt/mut	wt/mut
N315	Newman	common	Product[a]	T0[b]	T30[b]
SA0084	NWMN_0029		hypothetical protein	4.4	3.1
SA0098	NWMN_0047		similar to aminoacylase	2.0	2.4
SA0105	NWMN_0053		hypothetical protein	2.2	1.8
SA0144	NWMN_0095	*capA*	capsular polysaccharide synthesis enzyme Cap5A	4.5	7.0
SA0145	NWMN_0096	*capB*	capsular polysaccharide synthesis enzyme Cap5B	4.6	6.7
SA0146	NWMN_0097	*capC*	capsular polysaccharide synthesis enzyme Cap8C	4.5	7.5
SA0147	NWMN_0098	*capD*	capsular polysaccharide synthesis enzyme Cap5D	3.8	7.3
SA0148	NWMN_0099	*capE*	capsular polysaccharide synthesis enzyme Cap8E	2.6	6.2
SA0149	NWMN_0100	*capF*	capsular polysaccharide synthesis enzyme Cap5F	2.9	7.5
SA0150	NWMN_0101	*capG*	capsular polysaccharide synthesis enzyme Cap5G	2.8	7.8
SA0151	NWMN_0102	*capH*	capsular polysaccharide synthesis enzyme O-acetyl transferase Cap5H	3.1	8.6
SA0152	NWMN_0103	*capI*	capsular polysaccharide synthesis enzyme Cap5I	2.0	5.1

SA0153	NWMN_0104	*capJ*	capsular polysaccharide synthesis enzyme Cap5J	2.1	4.6
SA0154	NWMN_0105	*capK*	capsular polysaccharide synthesis enzyme Cap5K	2.1	3.5
SA0155	NWMN_0106	*capL*	capsular polysaccharide synthesis enzyme Cap5L	2.0	3.5
SA0156	NWMN_0107	*capM*	capsular polysaccharide synthesis enzyme Cap5M	2.1	3.8
SA0157	NWMN_0108	*capN*	capsular polysaccharide synthesis enzyme Cap5N	2.3	3.9
SA0158	NWMN_0109	*capO*	capsular polysaccharide synthesis enzyme Cap8O	1.9	3.7
SA0163	NWMN_0114		similar to cation-efflux system membrane protein CzcD	2.5	2.4
SA0181	NWMN_0131	*entB*	similar to isochorismatase	2.5	3.1
SA0182	NWMN_0132	*ipdC*	similar to indole-3-pyruvate decarboxylase	2.2	2.6
SA0208	NWMN_0153		maltose/maltodextrin transport permease homologue	2.2	2.2
SA0209	NWMN_0154		maltose/maltodextrin transport permease homologue	2.9	2.3
SA0210	NWMN_0155	*gatC*	similar to NADH-dependent dehydrogenase	2.8	2.4
SA0238	NWMN_0182		probable PTS galacitol-specific enzyme IIC component	2.5	2.0
SA0239	NWMN_0183		sorbitol dehydrogenase	2.9	2.3
SA0317	NWMN_0321		similar to dihydroflavonol-4-reductase	2.8	2.4
SA0318	NWMN_0322		similar to transport protein SgaT	8.1	2.5
SA0326	NWMN_0330		conserved hypothetical protein	2.4	2.4
SA0327	NWMN_0331		conserved hypothetical protein	2.0	2.3
SA0360	NWMN_0366		conserved hypothetical protein	2.3	2.8
SA0400	NWMN_0404	*lpl2nm*	staphylococcal tandem lipoprotein	1.9	2.4
SA0528	NWMN_0533		similar to hexulose-6-phosphate synthase	2.3	2.7
SA0529	NWMN_0534		conserved hypothetical protein	2.5	2.9
SA0531	NWMN_0536	*proP*	proline/betaine transporter homologue	1.9	2.0
SA0534	NWMN_0539	*vraB*	acetyl-CoA c-acetyltransferase	2.2	2.3
SA0549	NWMN_0555	*mvaK2*	phosphomevalonate kinase	2.4	2.1
SA0658	NWMN_0672		similar to plant-metabolite dehydrogenases	2.1	2.1
SA0659	NWMN_0673		similar to CsbB stress response protein	2.6	3.1
SA0665	NWMN_0679		coenzyme PQQ synthesis homologue	2.0	1.8
SA0666	NWMN_0680		6-pyruvoyl tetrahydrobiopterin synthase homologue	2.3	2.0
SA0721	NWMN_0734		conserved hypothetical protein	2.5	2.1
SA0722	NWMN_0735		conserved hypothetical protein	2.1	1.9
SA0740	NWMN_0754		hypothetical protein	2.8	2.5
SA0741	NWMN_0755		conserved hypothetical protein	2.0	2.1
SA0751	NWMN_0766		hypothetical protein	1.9	2.1
SA0755	NWMN_0771		similar to general stress protein 170	3.0	4.1
SA0760	NWMN_0776		glycine cleavage system protein H homologue	1.8	2.1
SA0768	NWMN_0778		conserved hypothetical protein	4.0	3.3
SA0780	NWMN_0791		similar to hemolysin	3.0	2.8
SA0781	NWMN_0792		similar to 2-nitropropane dioxygenase	3.2	2.3
SA0802	NWMN_0811		similar to NADH dehydrogenase	1.9	2.2
SA0830	NWMN_0840		conserved hypothetical protein	2.3	2.7
SA0848	NWMN_0859	*oppF*	oligopeptide transport system ATP-binding protein OppF homologue	2.3	1.8
SA0849	NWMN_0860		hypothetical protein, similar to peptide binding protein OppA	2.7	2.0
SA0905	NWMN_0922	*atl*	autolysin (N-acetylmuramyl-L-alanine	2.2	2.9

			amidase and endo-b-N-acetylglucosaminidase)		
SA1155	NWMN_1230		cardiolipin synthetase homolog	2.2	2.1
SA1184	NWMN_1263	*citB*	aconitate hydratase	2.3	2.4
SA1218	NWMN_1297	*pstB*	phosphate ABC transporter, ATP-binding protein (pstB)	2.0	3.1
SA1219	NWMN_1298		similar to phosphate ABC transporter	1.8	3.7
SA1365	NWMN_1439		glycine dehydrogenase (decarboxylating) subunit 2 homolog	2.1	2.7
SA1366	NWMN_1440		glycine dehydrogenase (decarboxylating) subunit 1	2.0	2.3
SA1367	NWMN_1441		aminomethyltransferase	2.2	2.4
SA1432	NWMN_1505		conserved hypothetical protein	6.7	4.1
SA1433	NWMN_1506		conserved hypothetical protein	5.1	2.6
SA1435	NWMN_1508		similar to acetyl-CoA carboxylase (biotin carboxyl carrier subunit), *accB* homolog	3.6	2.1
SA1436	NWMN_1509		conserved hypothetical protein	3.9	2.2
SA1443	NWMN_1516		conserved hypothetical protein	2.1	2.5
SA1517	NWMN_1587	*citC*	isocitrate dehyrogenase	3.0	4.5
SA1518	NWMN_1588	*citZ*	citrate synthase II	3.4	3.5
SA1528	NWMN_1600		conserved hypothetical protein	2.0	3.7
SA1529	NWMN_1601		conserved hypothetical protein	1.9	2.0
SA1557	NWMN_1629	*ccpA*	catabolite control protein A	43.8	73.9
SA1606	NWMN_1678		plant metabolite dehydrogenase homolog	1.9	2.1
SA1692	NWMN_1767		conserved hypothetical protein	2.4	2.7
SA1733	NWMN_1855		conserved hypothetical protein	2.8	2.2
SA1774	NWMN_1896		hypothetical protein [Bacteriophage phiN315]	2.0	1.9
SA1814	NWMN_1929		similar to succinyl-diaminopimelate desuccinylase	3.2	3.4
SA1844	NWMN_1946	*agrA*	accessory gene regulator A	2.3	1.8
SA1924	NWMN_2026		similar to aldehyde dehydrogenase	2.9	2.2
SA1946	NWMN_2048		conserved hypothetical protein	2.2	2.3
SA1981	NWMN_2080		conserved hypothetical protein	2.6	2.2
SA2006	NWMN_2109		similar to MHC class II analog	3.7	6.2
SA2082	NWMN_2188	*ureA*	urease gamma subunit	2.7	3.5
SA2083	NWMN_2189	*ureB*	urease beta subunit	3.8	4.2
SA2084	NWMN_2190	*ureC*	urease alpha subunit	2.3	2.6
SA2086	NWMN_2192	*ureF*	urease accessory protein UreF	2.0	2.1
SA2119	NWMN_2229		similar to dehydrogenase	2.3	2.4
SA2125	NWMN_2235		similar to formiminoglutamase	2.6	2.8
SA2170	NWMN_2282		similar to general stress protein 26	1.8	2.1
SA2204	NWMN_2315		phosphoglycerate mutase, pgm homolog	2.5	1.8
SA2220	NWMN_2331		conserved hypothetical protein	2.1	1.9
SA2243	NWMN_2353		similar to ABC transporter (ATP-binding protein)	2.0	2.1
SA2260	NWMN_2369		similar to glucose 1-dehydrogenase	2.9	2.5
SA2262	NWMN_2371		conserved hypothetical protein	2.8	2.8
SA2267	NWMN_2376		hypothetical protein	2.3	2.2
SA2309	NWMN_2419		conserved hypothetical protein	1.9	2.2
SA2331	NWMN_2442		hypothetical protein	1.9	2.3
SA2350	NWMN_2463		conserved hypothetical protein	2.6	1.8
SA2366	NWMN_2479		conserved hypothetical protein	3.0	3.1
SA2367	NWMN_2480		conserevd hypothetical protein	2.6	3.0
SA2374	NWMN_2487		conserved hypothetical protein	1.9	2.1
SA2479	NWMN_2585		conserved hypothetical protein	2.0	3.0
SA2485	NWMN_2591		hypothetical protein	3.4	3.6
SA2488	NWMN_2594		hypothetical protein	2.6	1.8
SAS022	NWMN_0777		truncated conserved hypothetical protein	2.0	2.4
SAS023	NWMN_0779		similar to thioredoxin	3.5	2.8
SAS056	NWMN_1861		hypothetical protein	2.1	2.5

[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website (18) and/or the KEGG website (29).
[b] Comparison of gene expression of wild type (wt) and Δ*ccpA* mutant (mut) at OD_{600} 1 (T0) and 30 min later (T30). Genes with a wt/mut ratio of ≤ 0.5 or ≥2 were considered to be regulated.

Supplementary Table 3: CcpA-dependent downregulation by glucose (factor of 0.5 or lower in wild type, factor of approximately 1, but below 2 in the mutant)

ID N315	Newman	common	Product[a]	wt +/-[b]	mut +/-[b]	*cre*[c]	Position
SA0008	NWMN_0007	*hutH*	histidine ammonia-lyase	0.5	1.1		
SA0016	NWMN_0016	*purA*	adenylosuccinate synthase	0.3	1.0		
SA0100	NWMN_0049		similar to Na+ Pi-cotransporter	0.2	1.7		
SA0107	NWMN_0055	*spa*	immunoglobulin G binding protein A precursor	0.2	1.1	TATTAAACCGCTTTCATT	-221 TTG
SA0162	NWMN_0113	*aldA*	aldehyde dehydrogenase homologue	0.3	1.0	AATGTAAACGCTTACTAT	- 87 ATG
SA0181	NWMN_0131	*entB*	similar to isochorismatase	0.2	1.2		
SA0182	NWMN_0132	*ipdC*	similar to indole-3-pyruvate decarboxylase	0.1	1.0	ATTGTTAGCGTTTTCAGA	-81 ATG
SA0184	NWMN_0134		conserved hypothetical protein	0.3	1.2	AATGTAAGCGATTACACA	-46 TTG
SA0185	NWMN_0135		similar to glucokinase regulator protein	0.2	1.2		
SA0186	NWMN_0136		similar to sucrose phosphotransferase enzyme II	0.4	1.2		
SA0187	NWMN_0137		RpiR family transcriptional regulator	0.2	1.2		
SA0224	NWMN_0168		3-hydroxyacyl-CoA dehydrogenase FadB homolog	0.4	1.1		
SA0299	NWMN_0253		similar to carbohydrate kinase, PfkB family	0.6	1.7	AATGTAAGCGTTTACAAC	-76 ATG
SA0300	NWMN_0253		truncated hypothetical protein	0.6	1.5		
SA0301	NWMN_0254		conserved hypothetical protein	0.3	1.7		
SA0302	NWMN_0255		probable pyrimidine nucleoside transport protein	0.7	1.8		
SA0395	NWMN_0402		similar to functionally unknown protein	0.4	0.9		
SA0477	NWMN_0481		conserved hypothetical protein	0.3	1.3		
SA0478	NWMN_0482		conserved hypothetical protein	0.3	1.2		
SA0605	NWMN_619		similar to dihydroxyacetone kinase	0.6	1.0	TATGATAGCGCATTCATT	-44 ATG
SA0606	NWMN_620		conserved hypothetical protein	0.5	1.0		
SA0607	NWMN_0621		conserved hypothetical protein	0.5	1.0		
SA0620	NWMN_0634		secretory antigen SsaA homologue	0.4	0.9		

SA0748	NWMN_0762		hypothetical protein	0.5	1.2		
SA0749	NWMN_0763		hypothetical protein	0.4	1.3		
SA0760	NWMN_0776		glycine cleavage system protein H homologue	0.3	1.0	AATGTAAGCGTTTACTAA	-135 TTG
SA0818	NWMN_0827	*rocD*	ornithine aminotransferase	0.3	1.0	AATGTAAGGGTTTTCAAA	- 133 ATG
SA0819	NWMN_0828	*gudB*	NAD-specific glutamate dehydrogenase	0.2	1.1	TTTGTAAGGGCTTTAAAA TTTGAAAGCGAAATCATT	- 24 TTG overlaps TTG
SA0830	NWMN_0840		conserved hypothetical protein	0.5	1.2		
SA0841	NWMN_0851		truncated MHC class II analog protein	0.4	0.9	AATTATAGCTTTTACATT	-120 ATG
SA0905	NWMN_0922	*atl*	autolysin (N-acetylmuramyl-L-alanine amidase and endo-b-N-acetylglucosaminidase)	0.4	1.1		
SA0915	NWMN_0932	*folD*	FolD bifunctional protein	0.4	1.3		
SA1019	NWMN_1086		acetyltransferase, GNAT family protein	0.3	1.1		
SA1041	NWMN_1109	*pyrR*	pyrimidine operon repressor chainA	0.4	1.1		
SA1088	NWMN_1155	*sucC*	succinyl-CoA synthetase (beta subunit)	0.2	1.4		
SA1089	NWMN_1156	*sucD*	succinyl-CoA synthetase (alpha subunit)	0.2	1.5	TACAATAGCGCTTACATT	- 48 ATG
SA1140	NWNM_1207	*glpF*	glycerol uptake facilitator	1.3	0.8	ATTGACAACGCTTTCATA	-116 ATG
SA1141	NWMN_1208	*glpK*	glycerol kinase	0.4	0.9		
SA1149	NWMN_1216	*glnR*	glutamine synthetase repressor	0.4	1.2		
SA1150	NWMN_1217	*glnA*	glutamine synthetase	0.5	1.3		
SA1172	NWMN_1249		guanosine monophosphate reductase	0.5	0.8		
SA1184	NWMN_1263	*citB*	aconitate hydratase	0.1	1.1		
SA1244	NWMN_1325	*odhB*	dihydrolipoamide succinyltransferase	0.1	1.1		
SA1245	NWNM_1326	*odhA*	2-oxoglutarate dehydrogenase E1	0.5	1.2	ATTGTAAGCGTTTCAACA	- 19 ATG
SA1365	NWMN_1439		glycine dehydrogenase (decarboxylating) subunit 2	0.1	1.1		

			homolog				
SA1366	NWMN_1440		glycine dehydrogenase (decarboxylating) subunit 1	0.1	1.2		
SA1367	NWMN_1441		aminomethyltransferase	0.2	1.4		
SA1432	NWMN_1505		conserved hypothetical protein	0.1	1.0		
SA1433	NWMN_1506		LamB/YcsF family protein	0.1	1.1		
SA1434	NWMN_1507	*accC*	acetyl-CoA biotin carboxylase	0.1	1.0		
SA1435	NWMN_1508	*accB*	acetyl-CoA carboxylase, biotin carboxyl carrier	0.2	0.8		
SA1436	NWMN_1509		urea amidolyase-related protein	0.2	0.8		
SA1437	NWMN_1510		similar to allophanate hydrolase subunit 1	0.3	0.6	AATGAAAAGGTATTCAAT ATTGTATGCGATTTGAAT	-34 GTG -86 ATG
SA1516	NWMN_1586	*phoP*	alkaline phosphatase synthesis transcriptional regulatory protein	0.4	1.6		
SA1517	NWMN_1587	*citC*	isocitrate dehyrogenase	0.1	1.0		
SA1518	NWMN_1588	*citZ*	citrate synthase II	0.1	0.9	TGTGAAAGCCATTTCATA	-27 ATG
SA1528	NWMN_1600		universal stress protein family protein	0.3	1.0		
SA1531	NWMN_1603	*ald*	alanine dehydrogenase	0.1	1.0	TTTGATTGCGCTTTCAAA	-36 ATG
SA1553	NWMN_1625	*fhs*	formyltetrahydrofolate synthetase	0.4	1.1		
SA1609	NWMN_1681	*pckA*	phosphoenolpyruvate carboxykinase [ATP]	0.2	0.7	AATGTAAAGGCTTACATT	-27 ATG
SA1617	NWMN_1688		similar to functionally unknown protein	0.3	1.3	TTTAAAAACTTTTTCAAA	-274 ATG
SA1618	NWMN_1689		conserved hypothetical protein	0.4	1.3		
SA1848	NWMN_1950	*nrgA*	probabale ammonium transporter	0.4	0.8		
SA1889	NWMN_1990		hypothetical protein	0.4	1.0		
SA1890	NWMN_1991		conserved hypothetical protein	0.5	1.0		
SA1900	NWMN_2002		conserved hypothetical protein	0.4	1.0		
SA1932	NWMN_2034		similar to hypothetical protein T13D8.31 - Arabidopsis thaliana	0.5	0.8		

SA1938	NWMN_2040	*pdp*	pyrimidine nucleoside phosphorylase	0.5	1.0		
SA1968	NWMN_2065	*arg*	arginase	0.4	1.0	ATGGTAAGCGCATACATT	-35 ATG
SA2121	NWMN_2231	*hutI*	imidazolonepropionase	0.5	1.1		
SA2122	NWMN_2232	*hutU*	urocanate hydratase	0.7	1.3	TATGTAACCGCATACATA	-41 ATG
SA2226	NWMN_2337		amino acid permease	0.2	0.9		
SA2227	NWMN_2337		truncated hypothetical protein, similar to D-serine/D-alanine/glycine transporter	0.1	0.9		
SA2311	NWMN_2421		NAD(P)H-flavin oxidoreductase	0.5	1.0	ATTGCAAACGGATTACTT	-67 ATG
SA2341	NWMN_2454	*rocA*	1-pyrroline-5-carboxylate dehydrogenase	0.2	1.2	AATGAAAGCGATTGCAAA	-96 ATG
SA2353	NWMN_2466		similar to secretory antigen precursor SsaA	0.5	1.0		
SA2356	NWMN_2469	*isaA*	immunodominant antigen A	0.4	0.8		
SA2366	NWMN_2479		conserved hypothetical protein	0.4	1.1		
SA2367	NWMN_2480		conserevd hypothetical protein	0.4	1.0		
SA2378	NWMN_2491		conserved hypothetical protein	0.5	1.2		
SA2420	NWMN_2526	*phoB*	alkaline phosphatase (EC 3.1.3.1) III precursor	0.2	1.4		
SA2479	NWMN_2585		conserved hypothetical protein	0.5	1.3		
SA2485	NWMN_2591		hypothetical protein	0.4	1.4		
SAS022	NWMN_0777		truncated conserved hypothetical protein	0.4	1.1		
SAS068	NWMN_1989		hypothetical protein	0.5	0.9		
SAS074	NWMN_2074		conserved hypothetical protein	0.5	0.6	CTTGAAAACGATTACAAA	-47 ATG

[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website (19) and/or the KEGG website (31).
[b] Comparison of gene expression with (+) and without (-) glucose, genes with a +/- glucose ratio of ≤ 0.5 or ≥2 in the wild-type were considered to be regulated.
[c] *cre*-site according to (48), allowing up to two mismatches. Palindromic parts are underlined.

Supplementary Table 4: Genes with CcpA-dependent upregulation by glucose in the wild-type (factor of 2 or higher in wild type, factor of approximately 1, but above 0.5 in the mutant)

ID N315	Newman	common	Product[a]	wt +/-[b]	mut +/-[b]	*cre*[c]	Position
SA0009	NWMN_0008	*serS*	seryl-tRNA synthetase	2.6	0.8		
SA0143	NWMN_0094	*adhE*	alcohol-acetaldehyde dehydrogenase	2.3	1.2		
SA0166	NWMN_0116		similar to nitrate transporter	2.8	1.1		
SA0167	NWMN_0117		similar to membrane lipoprotein SrpL	2.8	1.6	TATGGAAACATTTGCATT	-50 ATG
SA0168	NWMN_0118		similar to probable permease of ABC transporter	2.3	1.1		
SA0214	NWMN_0158	*uhpT*	hexose phosphate transport protein	2.1	1.1		
SA0218	NWMN_0162	*pflB*	formate acetyltransferase	2.7	1.6	TATGAAAACGTTAACATA	-42 ATG
SA0219	NWMN_0163	*pflA*	formate acetyltransferase activating enzyme	2.3	1.6		
SA0230	NWMN_0174		conserved hypothetical protein	2.3	1.9		
SA0334	NWMN_0339		sec-independent protein translocase protein TatC	2.3	1.1		
SA0335	NWMN_0340		twin-arginine translocation protein TatA	2.2	1.4		
SA0373	NWMN_0378	*xprT*	xanthine phosphoribosyltransferase	5.3	1.1		
SA0374	NWMN_0379	*pbuX*	xanthine permease	7.2	1.1		
SA0375	NWMN_0380	*guaB*	inositol-monophosphate dehydrogenase	3.6	1.2		
SA0376	NWMN_0381	*guaA*	GMP synthase (glutamine-hydrolyzing)	3.3	1.2		
SA0411	NWMN_0418	*ndhF*	NADH dehydrogenase subunit 5	2.5	0.9		
SA0412	NWMN_0419		conserved hypothetical protein	2.6	1.1		
SA0452	NWMN_0457	*veg*	VEG protein homologue	3.1	1.0		
SA0461	NWMN_0466	*mfd*	transcription-repair coupling factor	2.1	1.1		
SA0462	NWMN_0467		similar to low temperature requirement B protein	2.6	1.2		
SA0492	NWMN_0496		hypothetical protein	2.4	1.0		

SA0496	NWMN_0500	*rplA*	50S ribosomal protein L1 (BL1)	2.1	0.9		
SA0497	NWMN_0501	*rplJ*	50S ribosomal protein L10 (BL5)	2.6	1.6		
SA0577	NWMN_0592		similar to FimE recombinase	2.0	1.3		
SA0622	NWMN_0636	*rbf*	similar to AraC/XylS family transcriptional regulator	2.2	1.0		
SA0655	NWMN_0669	*fruA*	fructose specific permease	2.4	1.3	ATTGAAAAAGCATTCCAA	-116 ATG
SA0685	NWMN_0699	*nrdI*	NrdI protein involved in ribonucleotide reductase function	2.1	1.0		
SA0686	NWMN_0700	*nrdE*	ribonuceloside diphosphate reductase major subunit	3.2	1.4		
SA0687	NWMN_0701	*nrdF*	ribonucleoside-diphosphate reductase minor subunit	1.9	1.1		
SA0726	NWMN_0740	*gapR*	glycolytic operon regulator	1.5	1.3		
SA0727	NWMN_0741	*gap*	glyceraldehyde-3-phosphate dehydrogenase	1.7	1.6		
SA0728	NWMN_0742	*pgk*	phosphoglycerate kinase	2.2	1.6		
SA0729	NWMN_0743	*tpi*	triosephosphate isomerase	2.0	1.3		
SA0730	NWMN_0744	*pgm*	2, 3-diphosphoglycerate-independent phosphoglycerate mutase	1.7	1.2		
SA0731	NWMN_0745	*eno*	enolase (2-phosphoglycerate dehydrogenase)	1.4	1.3		
SA0769	NWMN_0780		D-methionine transport system ATP-binding protein	2.4	0.8		
SA0770	NWMN_0780		D-methionine transport system permease	2.4	1.0		
SA0922	NWMN_0938	*purF*	phosphoribosylpyrophosphate amidotransferase PurF	2.7	1.2		
SA0923	NWMN_0939	*purM*	phosphoribosylformylglycinamidine cyclo-ligase PurM	3.2	1.1		
SA0924	NWMN_0940	*purN*	phosphoribosylglycinamide formyltransferase	3.2	0.9		
SA0925	NWMN_0941	*purH*	bifunctional purine biosynthesis protein PurH	3.9	0.9		
SA0926	NWMN_0942	*purD*	phosphoribosylamine-glycine ligase PurD	3.2	0.9		
SA0938	NWMN_0953	*cydB*	cytochrome D ubiquinol oxidase subunit II homolog	2.0	1.1		
SA1009	NWMN_1075		similar to exotoxin 1	2.2	0.8		
SA1010	NWMN_1076		similar to exotoxin 4	2.3	0.6		
SA1200	NWMN_1280	*trpG*	anthranilate synthase component II	2.3	1.0		
SA1201	NWMN_1281	*trpD*	anthranilate phosphoribosyltransferase	2.0	1.0		

SA1202	NWMN_1282	*trpC*	indole-3-glycerol phosphate synthase	2.0	0.9		
SA1203	NWMN_1283	*trpF*	phosphoribosylanthranilate isomerase	2.2	1.0		
SA1204	NWMN_1284	*trpB*	tryptophan synthase beta chain	1.8	1.0		
SA1205	NWMN_1285	*trpA*	tryptophan synthase alpha chain	1.4	1.0		
SA1240	NWMN_1321		conserved hypothetical protein	2.4	1.2		
SA1241	NWMN_1322		similar to nitric-oxide reductase	2.3	1.2		
SA1270	NWMN_1347		similar to amino acid permease	2.0	1.1		
SA1295	NWMN_1373		conserved hypothetical protein	2.0	1.2		
SA1339	NWMN_1415	*malR*	maltose operon transcriptional repressor	2.2	1.0	GTTGGAAGCGTTTCCAA TATGCAATCGTTGCACA	- 27 ATG -94 ATG
SA1442	NWMN_1515		similar to caffeoyl-CoA O-methyltransferase	2.0	0.8		
SA1493	NWMN_1563	*hemD*	uroporphyrinogen III synthase	2.5	1.1		
SA1494	NWMN_1564	*hemC*	porphobilinogen deaminase	2.8	0.9		
SA1495	NWMN_1565	*hemX*	HemA concentration negative effector *hemX*	2.7	0.9		
SA1502	NWMN_1572	*rplT*	50S ribosomal protein L20	1.9	0.8		
SA1503	NWMN_1573	*rpmI*	50S ribosomal protein L35	2.1	0.8		
SA1504	NWMN_1574	*infC*	translation initiation factor IF-3 infC	2.4	0.9		
SA1506	NWMN_1576	*thrS*	threonyl-tRNA synthetase 1	2.2	1.4		
SA1608	NWMN_1680	*metK*	S-adenosylmethionine synthetase	2.2	0.9	AATGTAAGCCTTTACATT	-225 ATG
SA1700	NWMN_1822	*vraR*	two-component response regulator	2.2	0.8		
SA1701	NWMN_1823	*vraS*	two-component sensor histidine kinase	2.5	0.7		
SA1702	NWMN_1824		conserved hypothetical protein	2.1	0.8		
SA1710	NWMN_1832		similar to DNA polymerase III, alpha chain PolC type	2.1	0.8		
SA1798	NWMN_1915		hypothetical protein (Bacteriophage phiN315)	2.0	1.7		

SA1849	NWMN_1951		conserved hypothetical protein	2.6	1.2		
SA1850	NWMN_1952		conserved hypothetical protein	2.1	1.3		
SA1870	NWMN_1971	*rsbW*	anti-sigmaB factor	2.2	1.1		
SA1960	NWMN_2057	*mtlF*	PTS system, mannitol specific IIBC component	6.4	0.2		
SA2053	NWMN_2158		glucose uptake protein homolog	2.5	1.2		
SA2183	NWMN_2295	*narJ*	similar to nitrate reductase delta chain	2.1	0.7		
SA2184	NWMN_2296	*narH*	nitrate reductase beta chain narH	2.4	0.7		
SA2186	NWMN_2298	*nasF*	uroporphyrin-III C-methyl transferase	3.2	0.9		
SA2187	NWMN_2299	*nasE*	assimilatory nitrite reductase	3.5	1.0		
SA2188	NWMN_2300	*nasD*	nitrite reductase	2.4	0.9		
SA2234	NWMN_2344	*opuCD*	probable glycine betaine/carnitine/choline ABC transporter (membrane part) OpuCD	1.6	1.2		
SA2235	NWMN_2345	*opuCC*	glycine betaine/carnitine/choline ABC transporter (osmoprotection) OpuCC	1.9	1.2		
SA2236	NWMN_2346	*opuCB*	probable glycine betaine/carnitine/choline ABC transporter (membrane part) OpuCB	1.9	1.1		
SA2237	NWMN_2347	*opuCA*	glycine betaine/carnitine/choline ABC transporter (ATP-binding) OpuCA	2.6	1.0	AATAAATTCGCTTTTAAA	-311 ATG
SA2239	NWMN_2349		similar to amino acid transporter	2.2	1.1		
SA2290	NWMN_2397	*fnbB*	fibronectin-binding protein homolog	2.6	0.9		
SA2329	NWMN_2440	*cidA*	conserved hypothetical protein	3.5	1.4	TATGGAAACGCTCTCTAA	-107 ATG
SA2443	NWMN_2549		similar to accessory secretory protein Asp3	2.0	1.2		
SA2444	NWMN_2550		similar to accessory secretory protein Asp2	2.3	1.3		
SAS091	NWMN_2600		hypothetical protein	9.7	1.9		

[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website (19) and/or the KEGG website (31).
[b] Comparison of gene expression with (+) and without (-) glucose, genes with a +/- glucose ratio of ≤ 0.5 or ≥2 in the wild-type were considered to be regulated.
[c] *cre*-site according to (48), allowing up to two mismatches. Palindromic parts are underlined.

Supplementary Table 5: Glycolysis/Gluconeogenes-associated genes

ID				wt	mut
N315	Newman	common	Product[a]	+/-[b]	+/-[b]
SA0727	NWMN_0741	*gap*	glyceraldehyde-3-phosphate dehydrogenase	1.7	1.6
SA0728	**NWMN_0742**	***pgk***	**phosphoglycerate kinase**	**2.2**	**1.6**
SA0729	**NWMN_0743**	***tpi***	**triosephosphate isomerase**	**2.0**	**1.3**
SA0730	NWMN_0744	*pgm*	phosphoglycerate mut	1.7	1.2
SA0731	NWMN_0745	*eno*	enolase (2-phosphoglycerate dehydrogenase)	1.4	1.3
SA0823	NWNM_0833	*pgi*	glucose-6-phosphate isomerase A	0.9	1.2
SA0943-1	NWMN_0959	*pdhA*	pyrubate dehydrogenase E1 component alpha subunit	1.1	1.6
SA0944	NWMN_0960	*pdhB*	pyruvate dehydrogenase E1 component beta subunit	0.9	1.6
SA0945	NWMN_0961	*pdhC*	dihydrolipoamide S-acetyltransferase component of pyruvate dehydrogenase complex E2	0.7	1.4
SA0946	NWMN_0962	*pdhD*	dihydrolipoamide dehydrogenase component of pyruvate dehydrogenase E3	0.7	1.1
*SA1510	NWMN_1580	*gapB*	glyceraldehyde 3-phosphate dehydrogenase 2	no data	no data
SA1520	NWMN_1592	*pykA*	pyruvate kinase	1.6	1.1
SA1521	NWNM_1593	*pfk*	6-phosphofructokinase	0.6	1.1
***SA1609**	**NWNM_1681**	***pckA***	**phosphoenolpyruvate carboxykinase**	**0.2**	**0.7**
SA1927	NWMN_2029	*fbaA*	fructose-bisphosphate aldolase	1.5	1.1
SA2304	NWMN_2414	*fbp*	fructose-bisphosphatase	1.0	0.6

[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website (19) and/or the KEGG website (31).
[b] Comparison of gene expression with (+) and without (-) glucose, genes with a +/- glucose ratio of ≤ 0.5 or ≥2 in the wild-type were considered to be regulated.
* Genes with *cre*-sites.
Genes in **bold letters** are regulated.

Supplementary Table 6: TCA-associated genes

ID				wt	mut
N315	Newman	common	Product[a]	+/-[b]	+/-[b]
SA0545	NWMN_0550	*pta*	phosphotransacetylase	1.1	1.2
*SA0963	NWNM_0979	*pycA*	pyruvate carboxylase	0.9	0.7
SA0994	NWNM_2428	*sdhC*	succinate dehydrogenase cytochrome b-558	1.1	0.9
SA0995	NWNM_2429	*sdhA*	succinate dehydrogenase flavoprotein subunit	0.8	1.0
SA0996	**NWNM_2430**	***sdhB***	**succinate dehydrogenase iron-sulfur protein subunit**	**0.5**	**1.0**
SA1088	**NWNM_1155**	***sucC***	**succinyl-CoA synthetase (beta subunit)**	**0.2**	**1.4**

SA1089	**NWNM_1156**	***sucD***	**succinyl-CoA synthetase (alpha subunit)**	**0.2**	**1.5**
SA1184	**NWNM_1263**	***citB***	**aconitate hydratase**	**0.1**	**1.1**
SA1244	**NWNM_1325**	***odhB/ sucB***	**dihydrolipoamide succinyltransferase**	**0.1**	**1.1**
SA1245	**NWNM_1326**	***odhA/ sucA***	**2-oxoglutarate dehydrogenase E1**	**0.5**	**1.2**
SA1517	**NWNM_1587**	***citC***	**isocitrate dehyrogenase**	**0.1**	**1.0**
SA1518	**NWNM_1588**	***citZ***	**citrate synthase II**	**0.1**	**0.9**
SA1533	NWMN_1605	*ackA*	acetate kinase homolog	1.3	1.1
SA1554	NWMN_1626	*acsA*	acetyl-CoA synthetase	0.8	0.9
SA1669	NWNM_1743	*citG/ fumC*	fumarate hydratase, class-II	0.6	1.1

[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website (19) and/or the KEGG website (31).
[b] Comparison of gene expression with (+) and without (-) glucose, genes with a +/- glucose ratio of ≤ 0.5 or ≥2 in the wild-type were considered to be regulated.
* Genes with *cre*-sites.
Genes in **bold letters** are regulated.

Supplementary Table 7: Genes of amino acid degradation regulated by CcpA

ID				wt	mut
N315	Newman	common	Product[a]	+/-[b]	+/-[b]
SA0008	NWMN_0007	*hutH*	histidine ammonia-lyase	0.5	1.1
*SA0162	NWMN_0113	*aldA*	aldehyde dehydrogenase homologue	0.4	1.1
*SA0818	NWMN_0827	*rocD*	ornithine aminotransferase	0.3	1.0
*SA0819	NWMN_0828	*gudB*	NAD-specific glutamate dehydrogenase	0.2	1.1
SA1150	NWMN_1217	*glnA*	glutamine synthetase	0.5	1.3
SA1365	NWMN_1439		glycine dehydrogenase (decarboxylating) subunit 2	0.1	1.1
SA1366	NWMN_1440		glycine dehydrogenase (decarboxylating) subunit 1	0.1	1.2
SA1367	NWMN_1441		aminomethyltransferase	0.2	1.4
SA1531	NWMN_1603	*ald*	alanine dehydrogenase	0.1	1.0
*SA1968	NWMN_2065	*arg(rocF)*	arginase	0.4	1.0
SA2121	NWMN_2231	*hutI*	imidazolonepropionase	0.5	1.1
*SA2341	NWMN_2454	*rocA*	1-pyrroline-5-carboxylate dehydrogenase	0.2	1.2

[a] Cellular main roles are in accordance with the N315 annotation of the DOGAN website (19) and/or the KEGG website (31).
[b] Comparison of gene expression with (+) and without (-) glucose, genes with a +/- glucose ratio of ≤ 0.5 or ≥2 in the wild-type were considered to be regulated.
* Genes with *cre*-sites.

4 Authors' contribution

Project 1 - *Staphylococcus aureus* CcpA affects virulence determinant production and antibiotic resistance

Kati Seidl, Martin Stucki, Martin Rüegg, Christiane Goerke, Christiane Wolz, Llinos Harris, Brigitte Berger-Bächi, and Markus Bischoff

KS generated strain KS30, performed all physiological measurements, Northern blot analyses, population analysis profiles, and contributed to writing the manuscript. MS constructed the Δ*ccpA* mutants of strain Newman and COL, the complementing plasmid pMST1, determined CFUs and conducted several preliminary experiments. MR constructed plasmid pMR2. CG and CW performed RT-PCR and indirect immunofluorescence assays. LH performed electron microscopy. MB participated in the design of the study and drafted the manuscript. BBB was group leader, organized the funding, designed the study and participated in writing of the manuscript.

Project 2 - *Staphylococcus aureus* CcpA affects biofilm formation

Kati Seidl, Christiane Goerke, Christiane Wolz, Dietrich Mack, Brigitte Berger-Bächi, and Markus Bischoff

KS performed biofilm assays, stability assays, primary adherence assays, PIA-detection, Northern blot analyses, autolysis assays, participated in the design of the study, contributed to the interpretation of the results, and drafted the manuscript. CG and CW performed RT-PCR experiments. DM helped with the PIA-detection. MB participated in the design of the study, in writing of the manuscript. BBB was group leader, organized the funding, and participated in the design of the study and in writing of the manuscript.

Project 3 – *Staphylococcus aureus* CcpA controls *tst* expression

Kati Seidl, Markus Bischoff, and Brigitte Berger-Bächi

KS performed all experiments, participated in the design of the study, contributed to the interpretation of the results, and drafted the manuscript. MB participated in the design of the study and in writing of the manuscript. BBB was group leader, organized the funding, participated in the design of the study, and in writing of the manuscript.

Project 4 - The role of CcpA in the metabolism of *Staphylococcus aureus*

Kati Seidl, Patrice François, Jacques Schrenzel, Markus Bischoff, and Brigitte Berger-Bächi

KS experimentally validated the microarray data, performed computational analyses of *cre*-sites, Northern blot analyses, urease assays, contributed to the interpretation of the results, and drafted the manuscript. PF of the group of JS carried out the microarrays and performed statistical analyses. MB and BBB conceived, and coordinated the study. In addition, BBB was group leader, organized funding and participated in writing the manuscript.

5 Comments – Outlook

The results of this study suggest that the *S. aureus* CcpA directly links virulence and resistance to carbohydrate utilization and make it an interesting target for further investigation.

Our findings, that CcpA regulates the expression of *fnbB*, *spa*, *isaA,* the *cap*-genes and also biofilm formation reveal a possible role in host-pathogen interactions. This aspect could be further investigated *in vitro* by using matrix proteins or different host cell types to perform adhesion and internalization studies. In addition, *in vivo* assays could be carried out, employing various invertebrate and vertebrate animal models of infection. Of particular interest would be the use of diabetic mice. *S. aureus* is the predominant pathogen in foot infections of diabetic patients and blood glucose levels in diabetic patients often exceed 10 mM, the amount of glucose we found to cause CcpA-dependent regulation of many *S. aureus* genes.

It still remains unclear, why the *agr*-negative strain N315 exhibited such weak *tst* expression that was mostly insensitive to glucose-induced stimuli, in contrast to the other strains tested. As this strain seems to possess normal CcpA activity, the reason might be the missing *RNAIII,* which is known to induce *tst*-transcription. Therefore, the impact of *RNAIII* on CcpA-mediated regulation of *tst* needs to be investigated in more detail. Strain N315 also differs in terms of metabolism from other strains by being unable to catabolize acetate and having a truncated CcpC homologue, a protein known to regulate genes of the TCA cycle in *B. subtilis*. These aspects may have unknown consequences on *tst* expression, and also need further investigation.

Analysis of the CcpA-regulon showed that in addition to the CcpA-mediated, glucose-induced regulation, an even higher number of genes was regulated by CcpA in a glucose-independent way. A subject of further work could therefore include a closer investigation of the mechanisms by which this regulation is exerted.

Our expression analysis focussed on the short term effects of glucose, triggered within approximately one generation. Possible secondary effects mediated by regulators, which are under the control of CcpA might only be seen after longer exposures to glucose. In addition, our analysis was carried out in the exponential growth phase, and CcpA-regulated genes which are expressed at later stages could not be identified. To extend the list of CcpA-regulated genes, further microarray analyses could be carried out over time. To avoid secondary effects through changes in pH, glucose or oxygen levels, these investigations should ideally be performed under controlled conditions, using a fermenter. The data obtained could be combined with data from proteome analysis. To gain even more knowledge about the role of CcpA in the intermediary metabolism of *S. aureus,* ^{13}C-tracer

experiments using wild type and Δ*ccpA* mutant could be carried out. So far, the development of an appropriate amino acid-free medium, which is needed for this method, was not successful and needs further investigation.

Until now, the *cre*-consensus in *S. aureus* was assumed to be similar to the consensus sequences published for *B. subtilis*. To confirm this hypothesis or to find a more appropriate consensus for *S. aureus*, binding and/or foot printing assays could be carried out. This would imply the establishment of a functional *in vitro* system, including CcpA, HPr and a functional HPr kinase. This system might also be used for ChIP-on-chip, a technique which allows genome-wide location analysis of DNA sequences occupied by DNA-binding proteins, such as CcpA.

6 Appendix

6.1 Acknowledgements

I would like to thank the following persons:

Prof. Dr. Brigitte Berger-Bächi, who was group leader, organized the funding and made the whole project possible. She supported me in many ways, especially by revising this work.

Prof. Dr. Leo Eberl, my faculty supervisor, for his organizational help and support.

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Dr. Markus Bischoff, who gave me a guideline, and who also helped in the revision of this work.

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Miriam Ender for showing me everything when I was new in the BB lab and also for all the nice early-morning conversations and coffee-breaks.

Everybody else from the BB lab for any kind of help and support, and for making lab life worth living, even in bad times, when nothing seemed to work.

My parents for always being there and for making this work possible through financial support throughout my education.

My friends, who never forgot me, even in times in which I had disappeared to spend days and nights in the lab.

My own little family, including the baby in my belly, for supporting me in many ways.

6.2 Curriculum vitae

Personal data

Name	Seidl
First Name	Kati
Date of Birth	07 May 1980
Place of Birth	Chemnitz, Germany
Swiss Citizen of	Wattwil, SG

Education

2005-2008	Doctoral work at the Institute of Medical Microbiology, University of Zurich
2004	Graduation in Microbiology, University of Zurich
2001-2004	Studies in Microbiology, University of Zurich
1999-2001	1st and 2nd pre-degree in Biology, University of Fribourg
1998	Matura Typus E (A-levels), Kantonsschule Wattwil

Publications

2008 Berger-Bächi, B., M. Senn, M. Ender, **K. Seidl**, J. Hübscher, B. Schulthess, R. Heusser, P. Stutzmann-Meier, N. McCallum. Resistance to β-lactam antibiotics. *In* Archer G. L. (ed.), The staphylococci in human disease, 2nd ed. Blackwell publ. *In press.*

2008 **Seidl, K.**, C. Goerke, C. Wolz, D. Mack, B. Berger-Bächi, and M. Bischoff. The *Staphylococcus aureus* CcpA affects biofilm formation. Infection and Immunity **76:**2044-2055.

2007 Meier, S., C. Goerke, C. Wolz, **K. Seidl**, D. Homerova, B. Schulthess, J. Kormanec, B. Berger-Bächi, and M. Bischoff. 2007. σ^B and the σ^B-dependent *arlRS* and *yabJ-spoVG* loci affect capsule formation in *Staphylococcus aureus*. Infection and Immunity **75:**4562-4571.

2006 **Seidl, K.**, M. Stucki, M. Rüegg, C. Goerke, C. Wolz, L. Harris, B. Berger-Bächi, and M. Bischoff. 2006. *Staphylococcus aureus* CcpA affects virulence determinant production and antibiotic resistance. Antimicrobial Agents and Chemotherapy **50:**1183-1194.

6.3 Additional publications

σ^B and the σ^B-dependent *arlRS* and *yabJ-spoVG* loci affect capsule formation in *Staphylococcus aureus*

Collaboration

Most clinical isolates of *S. aureus* are able to produce extracellular capsular polysaccharides (CPs). CPs are thought to contribute to pathogenesis, by protecting *S. aureus* against opsonophagocytic killing by polymorphonuclear leukocytes and by enhancing virulence in a number of animal models of staphylococcal infection. Expression of CPs is known to be influenced by various environmental signals *in vitro* and *in vivo*, and transcription of the *cap* operon was shown to be modulated by various regulators. Recent microarray analyses showed that *capA* transcription was also under the control of the alternative σ-factor σ^B. The lack of an apparent σ^B consensus sequence in the promoter region of *capA* suggested that σ^B regulates *cap* transcription indirectly. This study showed that *capA* transcription and capsule formation were regulated by σ^B over the σ^B-modulated *arlRS* and *yabJ-spoVG* loci.

Contribution: Investigation of the effect of σ^B on *arlRS* expression, including Northern blot analyses, determination and interpretation of transcript sizes.

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σ^B and the σ^B-Dependent *arlRS* and *yabJ-spoVG* Loci Affect Capsule Formation in *Staphylococcus aureus*▽

Stefan Meier,[1] Christiane Goerke,[2] Christiane Wolz,[2] Kati Seidl,[1] Dagmar Homerova,[3] Bettina Schulthess,[1] Jan Kormanec,[3] Brigitte Berger-Bächi,[1] and Markus Bischoff[1,4]*

Institute of Medical Microbiology, University of Zürich, Zürich, Switzerland[1]; Institute for Medical Microbiology and Hygiene, University Hospital Tübingen, Tübingen, Germany[2]; Institute of Molecular Biology, Center of Excellence for Molecular Medicine, Slovak Academy of Sciences, Bratislava, Slovak Republic[3]; and Institute of Medical Microbiology and Hygiene, University of Saarland, Homburg, Germany[4]

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The alternative transcription factor σ^B of *Staphylococcus aureus* affects the transcription of the *cap* gene cluster, required for the synthesis of capsular polysaccharide (CP), although this operon is lacking an apparent σ^B-dependent promoter. Regulation of *cap* expression and CP production in *S. aureus* strain Newman was shown here to be influenced by σ^B, the two-component signal transduction regulatory system ArlRS, and the *yabJ-spoVG* locus to different extents. Inactivation of *arlR* or deletion of the *sigB* operon strongly suppressed *capA* (CP synthesis enzyme A) transcription. Deletion of *spoVG* had a polar effect on *yabJ-spoVG* transcription and resulted in a two- to threefold decrease in *capA* transcription. Interestingly, immunofluorescence showed that CP production was strongly impaired in all three mutants, signaling that the *yabJ-spoVG* inactivation, despite its only partial effect on *capA* transcription, abolished capsule formation. *trans*-Complementation of the Δ*spoVG* mutant with *yabJ-spoVG* under the control of its native promoter restored CP-5 production and *capA* expression to levels seen in the wild type. Northern analyses revealed a strong impact of σ^B on *arlRS* and *yabJ-spoVG* transcription. We hypothesize that ArlR and products of the *yabJ-spoVG* locus may serve as effectors that modulate σ^B control over σ^B-dependent genes lacking an apparent σ^B promoter.

Staphylococcus aureus is a major nosocomial pathogen with the ability to cause a variety of diseases, including life-threatening infections. Like most microorganisms that are able to cause invasive diseases, *S. aureus* produces extracellular capsular polysaccharides (CPs), which are thought to be of importance in pathogenesis (reviewed in reference 35). Although 11 serologically distinct CPs were identified in *S. aureus*, the majority of clinical isolates produce CPs of serotype 5 (CP-5) or serotype 8 (CP-8). CPs protect *S. aureus* against opsonophagocytic killing by polymorphonuclear leukocytes (16, 17, 25, 53, 56) and enhance virulence in a number of animal models of staphylococcal infection (34, 40, 53, 54, 57). Expression of CPs is known to be influenced by various environmental signals in vitro and in vivo (reviewed in references 35 and 56), and transcription of the *cap* operon was shown to be modulated by regulatory elements, such as *arlRS*, *agr*, *ccpA*, *mgr*, *sae*, and *sarA* (7, 8, 23, 24, 26, 27, 39, 48, 52, 55). Recent microarray analyses added the alternative σ factor σ^B to the regulatory network controlling *cap* operon expression (3, 38) and indicated σ^B to control *capA* transcription in a growth phase-dependent manner (3). However, the lack of an apparent σ^B consensus sequence in the promoter of *capA* suggested that σ^B regulates *cap* transcription indirectly. Candidates for such downstream-acting regulators might be ArlRS and SarA, which are positively controlled by σ^B in *S. aureus* (2, 3), although SarA was previously shown to have only a minor effect on *cap* expression and CP production in *S. aureus* (24). RNAIII of the *agr* locus, known to positively affect *capA* expression (7, 24, 55), could be excluded as a positive mediator of σ^B activity on *capA* transcription, since RNAIII is known to be repressed by σ^B activity (2, 3, 15). Further candidates for regulators mediating the effect of σ^B might be the putative regulator homologs YabJ and SpoVG (SaCOL0540/1), whose expression is also predominantly controlled by σ^B in *S. aureus* (3). *spoVG* was the first developmentally regulated gene cloned from spore-forming *Bacillus subtilis* (47). Mutations in *spoVG* were shown to impair spore formation of *B. subtilis* stationary-phase cells at stage V (44) and to enhance the stage II defect of a *spoIIB* mutation (29), leading to the assumption that SpoVG is involved in the formation of the spore cortex. More recent findings indicated the primary function of SpoVG to be in the regulation of asymmetric septation in stationary-phase cells (31). Based on the finding that close homologues of SpoVG of *B. subtilis* are present in the genomes of several nonsporulating bacteria as well, we recently suggested that the SpoVG homologues might fulfill different, more general regulatory functions in the latter group of bacteria (3). YabJ is a member of the highly conserved YigF protein family, which is represented in animals, fungi, and bacteria. Many biological processes were shown to be influenced by YigF proteins, but its exact biochemical function remains unknown, although the crystal structure of YabJ of *Bacillus subtilis* has been published (51).

Here, we show that σ^B and the σ^B-modulated *arlRS* and *yabJ-spoVG* loci reduced *capA* transcription and strongly impaired capsule formation in the CP-5-producing strain Newman.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this investigation are listed in Table 1. Bacteria were routinely grown in Luria-Bertani (LB) medium (Difco Laboratories, Detroit, MI) with aeration

* Corresponding author. Mailing address: Institute of Medical Microbiology, University of Zürich, Gloriastr. 32, 8006 Zürich, Switzerland. Phone: 41 44 634 26 70. Fax: 41 44 634 49 06. E-mail: Bischoff@immv.uzh.ch.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype[a]	Reference or source
Strains		
S. aureus		
RN4220	NCTC8325-4 r^- m^+ *rsbU*	21
COL	*mec*; high-Mc^r clinical isolate; Mc^r Tc^r	20
Newman	Clinical isolate (ATCC 25904); CP-5 producer	9
BF21	RN6390 *arlR*::*cat*; phenotypic *arlRS* mutant; Cm^r	10
GP268	NCTC8325 derivative; (*rsbU-V-W-sigB*)$^+$-*tet*(L); Tc^r	12
IK181	NCTC8325 derivative; Δ*rsbUVW-sigB*::*erm*(B); Erm^r	22
IK183	COL Δ*rsbUVW-sigB*::*erm*(B); Erm^r	22
IK184	Newman Δ*rsbUVW-sigB*::*erm*(B); Erm^r	22
SM1	RN4220 Δ*spoVG*::*erm*(B); phenotypic *yabJ-spoVG* mutant, Erm^r	This study
SM2	Newman Δ*spoVG*::*erm*(B); phenotypic *yabJ-spoVG* mutant, Erm^r	This study
SM99	Newman *arlR*::*cat*; Cm^r	This study
E. coli		
XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tc^r)]	Stratagene
Plasmids		
pAC7	Expression plasmid containing the P_{BAD} promoter and the *araC* gene; Cm^r	42
pAC7-*sigB*	pAC7 with a 0.75-kb PCR fragment of the *sigB* ORF from strain COL; Cm^r	14
pBT	1.6-kb PCR fragment of the *tet*(L) gene of pHY300PLK into Alw26I-digested pBC SK(+) (Stratagene); Tc^r	12
pBus1	*E. coli-S. aureus* shuttle plasmid with multicloning site from pBluescript II SK (Stratagene) and the *rrn*$T1_4$ terminator sequence from pLL2443; Tc^r	45
pEC1	pUC derivative containing the 1.45-kb ClaI *erm*(B) fragment of Tn*551*; Ap^r Em^r	4
pGC2	*E. coli-S. aureus* shuttle plasmid; Cm^r	Constructed and obtained by P. Matthews
pSA0455p	pSB40N with a 360-bp PCR fragment covering the σ^B-dependent promoter region preceding *yabJ-spoVG* fused to the reporter gene *lacZ*α; Ap^r	3
pSB40N	Promoter probe plasmid; Ap^r	19
pSP-*luc*$^+$	Firefly luciferase cassette vector; Ap^r	Promega
pSTM01	pEC1 with 0.5- and 1.1-kb PCR fragments covering the *spoVG* flanking regions; Em^r Ap^r	This study
pSTM02	pBT with a 3.1-kb KpnI/HindIII fragment of pSTM01 harboring the *spoVG* flanking regions and the *erm*(B) cassette fully replacing the *spoVG* coding region; Em^r Tc^r	This study
pSTM03	pSP-luc+ with a 0.4-kb PCR fragment covering the *capA* promoter region fused to the reporter gene *luc*$^+$; Ap^r Em^r	This study
pSTM04	pBus1 with a 2.1-kb KpnI/EcoRI fragment of pSTM03 harboring the *cap*A promoter-*luc*$^+$ fusion; Tc^r	This study
pSTM05	pGC2 with a 1-kb PCR fragment covering the σ^B-dependent *yabJ* promoter, *yabJ*, and *spOVG*; Cm^r	This study
pSTM06	pSB40N with a 0.8-kb PCR fragment covering the *arlRS* promoter fused to the reporter gene *lacZ*α; Ap^r	This study
pSTM07	pSB40N with a 0.4-kb PCR fragment covering the *capA* promoter region fused to the reporter gene *lacZ*α; Ap^r	This study
pSTM11	pBus1 with a 0.6-kb PCR fragment covering *yabJ* and the preceding σ^B-dependent promoter P_{yabJ}; Tc^r	This study
pSTM13	pBus1 with a 0.5-kb PCR hybrid including the σ^B-dependent promoter P_{yabJ} fused to the *spoVG* ORF; Tc^r	This study

[a] Abbreviations are as follows: Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Tc^r, tetracycline resistant.

(200 rpm) at 37°C. Where indicated, mutant strains were grown on antibiotic-supplemented media containing either 100 μg of ampicillin, 20 μg of chloramphenicol, 10 μg of erythromycin, or 10 μg of tetracycline per ml.

Strain construction. For the construction of an *S. aureus* Newman Δ*spoVG* mutant, DNA fragments covering 0.5 kb of the region upstream of *spoVG* (up fragment) and 1.1-kb of the region downstream of *spoVG* (down fragment) were amplified by PCR, using primer pairs oSTM01/02 and oSTM03/04, respectively, and *S. aureus* Newman DNA as the template (Table 2). The resulting PCR products were KpnI/BamHI and PstI/HindIII digested, respectively, and cloned into plasmid pEC1 (4), with the up fragment preceding the *erm*(B) cassette and the down fragment following the resistance marker. The resulting plasmid, pSTM01, was digested with KpnI and HindIII and the insert cloned into the suicide vector pBT (12), yielding plasmid pSTM02, which was electroporated into RN4220 (Fig. 1). Mutants with the allelic replacement were selected for erythromycin resistance and screened for loss of tetracycline resistance, yielding strain SM1 [RN4220 *spoVG*::*erm*(B)], which was subsequently used as a donor for transducing the *spoVG* deletion into the CP-5-producing *S. aureus* strain Newman, yielding strain SM2. The *spoVG* deletion in SM2 was confirmed by PCR and Southern analyses.

The Newman *arlR* mutant SM99 was constructed by transducing the *cat*-tagged *arlR* mutation of BF21 (10) into Newman and selecting for chloramphenicol resistance.

Plasmid construction. For the construction of the *capA* promoter-*luc*$^+$ reporter gene fusion plasmid pSTM04, a 0.4-kb DNA fragment covering the *capA* promoter region was amplified by PCR using primer pair capAp-F/capAp-R and *S. aureus* Newman DNA as the template (Table 2). The resulting PCR product was HindIII/NcoI digested and cloned into pSP-*luc*$^+$ (Promega) as a 5′ fusion to the reporter gene *luc*$^+$. A plasmid (pSTM03) harboring the promoter fragment was used to excise the promoter-reporter gene fragment by digestion with HindIII/XhoI, which was subsequently cloned into the multiple cloning site of

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]	Location[b] or reference
arlRprobe+	CAATGTGGACACAGAGTATG	1465970–1465989
arlRprobe−	CAGTTCGTGGCGTTGGG	1465422–1465438
arlSprobe+	CAGCAGTATTAGAAGAATCG	1464595–1464614
arlSprobe−	GAGTCCATTACCGCCTTGAC	1464154–1464173
Asp23probe+	ATGACTGTAGATAACAATAAAGC	11
Asp23probe−	TTGTAAACCTTGTCTTTCTTGG	11
capAp-F	cgcaagcttCAAACATCATATGATTATAAGC	153034–153055
capAp-R	gcgccatggTTTACCTCCCTTAAAAATT	153384–153402
oSTM01	taggtaccTCAAAAGAAGTTAAACAAAG	548665–548684
oSTM02	cggatccATATTAATCGAAAATTATAATTCC	549175–549198
oSTM03	cgcctgcagATTATGATGATATGAAAATTATTG	549706–549729
oSTM04	gcgaagcttGACCAATAACAACATCTTCGCC	550760–550781
oSTM20	GAAAATCATTAACACAACAAG	548806–548826
oSTM21	CTTAATTTTACTTACTAATTC	549155–549175
oSTM28	cgatggatccGTGTTATGAATTTAATGAATGAG	548603–548625
oSTM29	gcgtcgacTTATTGCAAATGTATTACATCGC	549574–549596
oSTM30	CTAAATAAAACAGAGAGATATATACTATAGG	549213–549243
oSTM31	gcgggtaccGTGTTATGAATTTAATGAATGAG	548603–548625
oSTM32	gcgggatccGAAGCTTGATTAAACATATTAATCG	549189–549213
oSTM33	gcgggatccTTATTGCAAATGTATTACATCGC	549574–549596
oSTM34	ggattttcatatgACTAAAACTCCTTTTATGAAAAC	548778–548800
oSTM61	cgcggatccCAAACATCATATGATTATAAGC	153034–153055
oSTM62	gcgctcgagTTTACCTCCCTTAAAAATT	153384–153402
oSTM63	cgcggatccGCAGTAAACCTAAAGTGTCG	1466817–1466836
oSTM64	gcgctcgagTTGTACACCTCATATTACGAC	1466067–1466087
oSTM71	gtgcatatgAAAGTGACAGATGTAAGACTTAG	549259–549281

[a] Lowercase letters represent nucleotide additions.
[b] Based on the sequence of strain COL (RefSeq accession no. NC_002951).

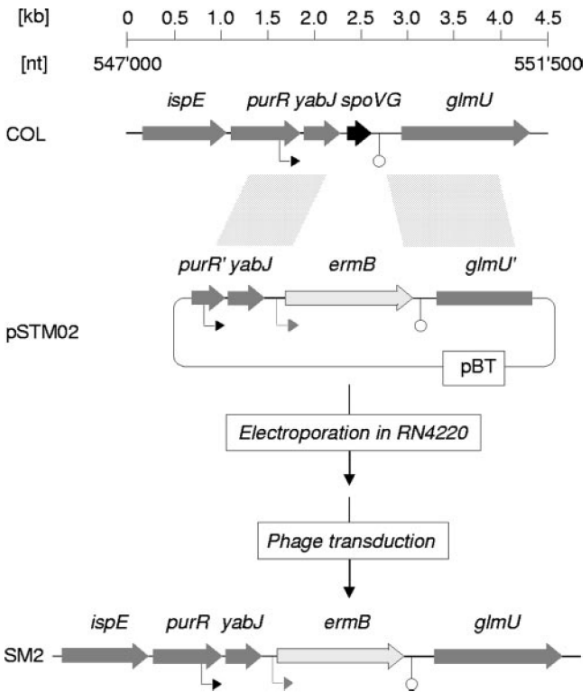

FIG. 1. Genetic organization of the *S. aureus yabJ-spoVG* locus. Schematic representations of the *yabJ-spoVG* region of *S. aureus* and of the strategy used to obtain SM2 are shown. ORFs, promoters, terminators, and regions allowing recombination are indicated. ORF notations and nucleotide (nt) numbers correspond to those of the respective genomic regions of strain COL (RefSeq accession no. NC_002951).

the *Escherichia coli-S. aureus* shuttle vector pBus1 (45) to obtain plasmid pSTM04. pSTM04 was finally used for electroporation of RN4220, from which it was transduced into strains Newman, SM2, SM99, and IK184 by phage transduction.

For the construction of pSTM05, a 995-bp DNA fragment covering the σ^B-dependent *yabJ* promoter, *yabJ*, and *spoVG* was amplified by PCR using primer pair oSTM28/oSTM29 and *S. aureus* Newman DNA as the template. The resulting PCR product was BamHI/SalI digested and cloned into the *E. coli-S. aureus* shuttle vector pGC2 (P. Matthews). pSTM05 was electroporated into RN4220, from which it was transduced into strains Newman and SM2.

For the construction of pSTM06 and pSTM07, DNA fragments representing 771 bp and 370 bp of the *arlR* and *capA* promoter regions of Newman, respectively, were generated by PCR using primer pairs oSTM63/oSTM64 and oSTM61/oSTM62. The PCR products were digested with BamHI and XhoI and cloned into promoter probe plasmid pSB40N (42) upstream of the *lacZα* reporter gene to obtain pSTM06 (*arlRp*) and pSTM07 (*capAp*).

For the construction of pSTM11, a 612-bp DNA fragment covering the σ^B-dependent *yabJ* promoter (P_{yabJ}) and *yabJ* was amplified by PCR using primer pair oSTM31/oSTM32 and *S. aureus* Newman DNA as the template. The resulting PCR product was BamHI/KpnI digested and cloned into the *E. coli-S. aureus* shuttle vector pBus1. For pSTM13 (P_{yabJ}-*spoVG*), harboring promoter P_{yabJ} fused to *spoVG*, primer pairs oSTM31/oSTM34 and oSTM71/oSTM33 were used together with pSTM05 to amplify 199- and 339-bp DNA fragments covering the σ^B-dependent promoter including the region preceding the *yabJ* open reading frame (ORF) and *spoVG*, respectively. The resulting PCR products were KpnI/NdeI and NdeI/BamHI digested and cloned into pBus1. pSTM11 and pSTM13 were electroporated into RN4220 and subsequently transduced into strains Newman and SM2.

Sequence analyses confirmed the identities of all cloned inserts.

Protease activity assay. The proteolytic activities of Newman, IK184, SM2, and SM99 were determined on casein agar plates as clear zones surrounding colonies.

Northern analyses. Overnight cultures of *S. aureus* were diluted 1:100 into fresh prewarmed LB medium and grown at 37°C and 200 rpm. Samples were removed from the culture at the time points indicated and centrifuged at 14,000 × *g* and 4°C for 1 min, the culture supernatants were discarded, and the cell sediments were snap frozen in liquid nitrogen. Total RNAs were isolated according to Cheung et al. (6). Blotting, hybridization, and labeling were performed as previously described (12). Primer pairs arlRprobe+/arlRprobe−, arlSprobe+/arlSprobe−, asp23probe+/asp23probe−, oSTM20/oSTM21, and oSTM29/oSTM30 were used to generate

digoxigenin-labeled *arlR*-, *arlS*-, *asp23*-, *yabJ*-, and *spoVG*-specific probes by PCR labeling.

Luciferase assays. Luciferase activity was measured as described earlier (2), using the luciferase assay substrate and a Turner Designs TD-20/20 luminometer (Promega).

LightCycler RT-PCR. The *capA* and *gyrB* transcripts were quantified by LightCycler reverse transcription (RT)-PCR as described earlier (48), using RNA samples obtained from cultures grown for 8 and 24 h in LB at 37°C and 200 rpm.

CP-5 determination. CP-5 production was determined by indirect immunofluorescence from cultures grown for 8 and 24 h in LB medium as described earlier (48), using mouse immunoglobulin M monoclonal antibodies to CP-5 (13). Quantification of CP-5-positive cells was done by determining the numbers of DAPI (4′,6′-diamidino-2-phenylindole)- and CY-3-positive cells using the program CellC 1.11 (Institute of Signal Processing, Tampere University of Technology, Finland).

Two-plasmid analysis. Testing of the interaction of *S. aureus* promoters with RNA polymerase containing *S. aureus* σ^B was done essentially as described earlier (14). The promoter-containing plasmids pSA0455p (*yabJ* promoter), pSTM06 (*arlR* promoter), and pSTM07 (*capA* promoter) were transformed into *E. coli* XL1Blue containing a compatible plasmid, either pAC7-*sigB* or empty pAC7. Clones were selected on LBACX-ARA plates (LB medium containing lactose [5 mg ml^{-1}], ampicillin [100 μg ml^{-1}], chloramphenicol [40 μg ml^{-1}], 5-bromo-4-chloro-3-indolyl-D-galactopyranoside [20 μg ml^{-1}], and arabinose [2 μg ml^{-1}]) and analyzed for color production (43).

RESULTS

Construction of SM2 and SM99. Inactivation of *spoVG* and *arlR* in *S. aureus* Newman yielded strains SM2 and SM99, respectively. Southern blots probed with *spoVG* or the C-terminal part of *arlR* confirmed the constructs (data not shown).

Transcriptional analysis of the *yabJ-spoVG* locus in Newman and SM2. Our recent microarray analyses suggested that *yabJ* and *spoVG* form a bicistronic operon that is predominantly controlled by σ^B activity (3). To test whether and how the deletion of *spoVG* affected the expression of the *yabJ-spoVG* locus, we analyzed the transcription of *yabJ* and *spoVG* in cells of Newman and SM2 during growth in LB. Our Northern analyses identified *yabJ*-specific signals with sizes of 1.2 and 0.6 kb and *spoVG*-specific signals with sizes of 1.2 and 0.5 kb in Newman. The 1.2-kb signal detected with both the *yabJ*- and the *spoVG*-specific probes appeared to be the major transcript of the *yabJ-spoVG* locus. It was present already after 1 h of growth and increased within time, while the minor 0.6-kb, *yabJ*-specific signal and the 0.5-kb, *spoVG*-specific signal appeared only after 3 h of growth (Fig. 2). In line with our Southern results, no *spoVG*-specific transcripts were detected in SM2. Based on our hypothesis that *yabJ* and *spoVG* are predominantly transcribed as a bicistronic mRNA and taking into account that the deletion of *spoVG* did not alter the genetic organization of *yabJ* and its promoter(s) in SM2, we expected the allelic replacement of *spoVG* with *ermB* to affect the sizes of the *yabJ* transcripts rather than the intensities of *yabJ* transcription. Surprisingly, we detected only a series of faint signals in our *yabJ* Northern analysis of SM2, indicating that the deletion of *spoVG* apparently had a polar effect on the expression of the preceding *yabJ*. We therefore considered SM2 to represent a phenotypic *yabJ-spoVG* mutant.

Phenotypic characterization of SM2 and SM99. The successful allelic replacement of *spoVG* and its impact on *yabJ* indicated that these genes, like *sigB* and *arlR*, were not essential for growth in vitro. Deletions of *sigB* and *arlR* were previously shown to affect the growth rates of the mutants (12, 23). While inactivation of *arlR* reduces growth during the early

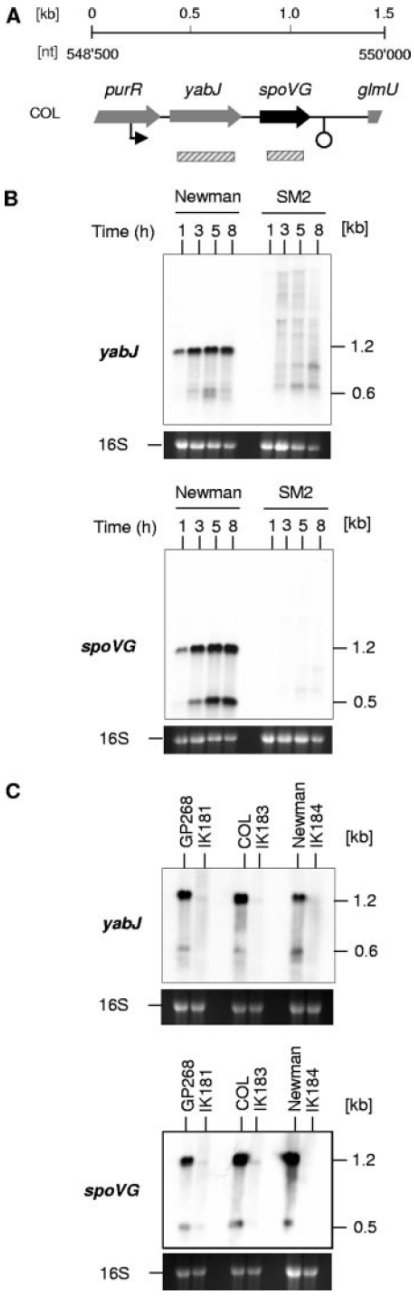

FIG. 2. Expression of *yabJ-spoVG* in *S. aureus*. (A) Schematic representation of the *yabJ-spoVG* region of *S. aureus* COL. ORFs, promoters, terminators, and the probes used are indicated. nt, nucleotide. (B) Northern blot analyses of the *yabJ* and *spoVG* transcriptions in Newman and SM2 (Δ*spoVG*) during growth in LB. (C) Effect of σ^B on *yabJ* and *spoVG* expression. Northern blot analyses of the *yabJ* and *spoVG* transcriptions in strains GP268, COL, and Newman and their isogenic Δ*rsbUVW-sigB* mutants after 8 h of growth. Relevant transcript sizes are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading.

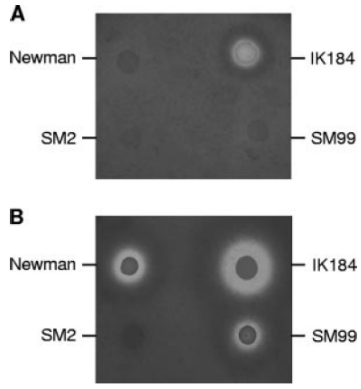

FIG. 3. Protease production of Newman, IK184 (Δ*rsbUVW-sigB*), SM2 (Δ*spoVG*), and SM99 (*arlR*) grown on casein agar plates for 24 h at 37°C (A) and then incubated for 72 h at room temperature (B).

stages (23), inactivation of *sigB* affects only the later stages of growth (12). Monitoring growth of strains Newman, SM2, and SM99 in LB over a period of 8 h confirmed the negative impact of the *arlR* mutation on the growth rate of SM99, while the growth curves of Newman and SM2 were virtually identical, indicating that deletion of *spoVG* was not associated with the growth defect observed for *sigB* mutants (data not shown). Inactivation of *sigB* is further known to prevent formation of staphyloxanthin (12, 22, 33), the orange end product of *S. aureus* carotenoid biosynthesis (30). Neither inactivation of *arlR* nor deletion of *spoVG* reduced pigment formation, indicating that ArlR and YabJ/SpoVG were not involved in the regulation of pigment production of *S. aureus* (data not shown). Mutations in *sigB* and *arlRS* are also known to affect the proteolytic activities of the mutants (10, 18, 23). We therefore tested the proteolytic activities of Newman and its derivatives IK184 (Δ*rsbUVW-sigB*), SM2 (Δ*spoVG*), and SM99 (*arlR*) on casein agar plates. After 24 h of incubation at 37°C, only IK184 produced a clear zone surrounding the colony, while neither Newman, SM2, nor SM99 exhibited such a clear zone (Fig. 3A). However, after storage of the incubated plates for 72 h at room temperature, clear zones surrounding colonies were also observed for Newman and SM99 but not for SM2 (Fig. 3B), suggesting that the inactivation of the *yabJ-spoVG* locus caused a suppression of extracellular protease production or activation in *S. aureus*.

Effect of σ^B on *arlRS* and *yabJ-spoVG* expression. We recently observed that *arlRS* transcription, like *yabJ-spoVG* transcription, is positively controlled by σ^B activity during later growth stages (3). While the positive effect of σ^B on *yabJ-spoVG* transcription was found in different *S. aureus* genetic lineages, including COL, GP268 (*rsbU*-positive NCTC8325 derivative), and Newman (3, 38), the positive impact of σ^B on *arlRS* expression seemed to be strain dependent and was seen only in *S. aureus* Newman so far (3). We therefore determined the *arlRS* and *yabJ-spoVG* expression patterns in cells of COL, GP268, Newman, and their isogenic Δ*rsbUVW-sigB* mutants grown in LB for 8 h. While clear *yabJ*- and *spoVG*-specific signals were visible in COL, GP268, and Newman at this growth stage, these transcripts were missing in all of the

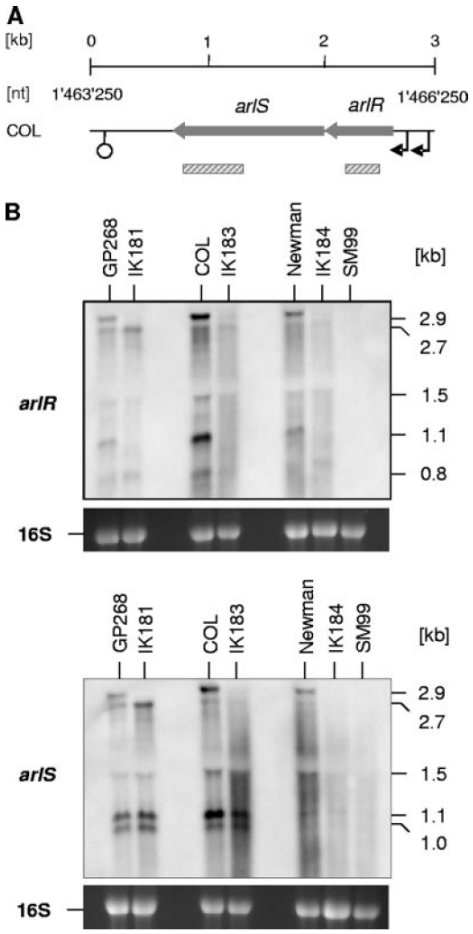

FIG. 4. Effect of σ^B on *arlRS* expression. (A) Schematic representation of the *arlRS* region of *S. aureus* COL. ORFs, promoters, terminators, and the regions used as probes are indicated. ORF notations and nucleotide (nt) numbers correspond to those of the respective genomic regions of strain COL (RefSeq accession no. NC_002951). (B) Northern blot analyses of the *arlR* and *arlS* transcriptions in strains GP268, COL, and Newman and their isogenic Δ*rsbUVW-sigB* mutants after 8 h of growth in LB at 37°C. Relevant transcript sizes and the probes used are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading.

Δ*rsbUVW-sigB* mutants (Fig. 2C), confirming the importance of σ^B activity for the expression of the *yabJ-spoVG* locus. Interestingly, our Northern analyses of *arlRS* transcription revealed a clear σ^B dependence in all genetic lineages analyzed (Fig. 4) and identified several new signals in a strain-dependent manner in addition to the 2.7- and 1.5-kb transcripts that have been reported for *arlRS* before (10). Hybridizing the total RNA samples with a probe specific for *arlR* produced signals with sizes of 2.9, 2.7, 1.5, 1.1, and 0.9 kb in all parental strains, while the 2.9- and 1.1-kb signals were missing in all Δ*rsbUVW-sigB* mutants. All signals were missing in the *arlR* mutant SM99, confirming that the signals were *arlR* specific. Hybridizing the same RNAs with a probe specific for *arlS* resulted in signals with sizes of 2.9, 2.7, and 1.5 kb in all parental strains,

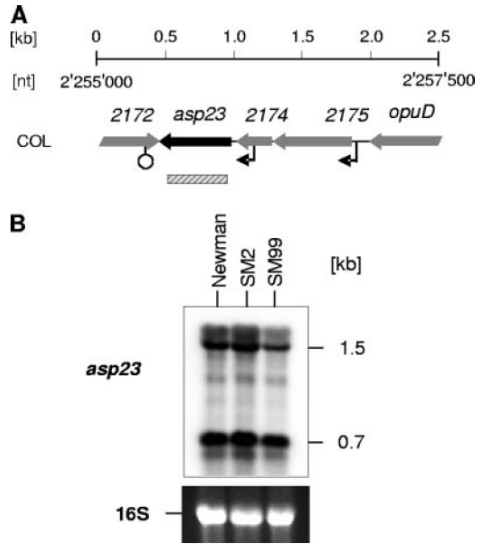

FIG. 5. Effect of *arlRS* and *yabJ-spoVG* on σ^B activity. (A) Schematic representation of the *asp23* region. ORFs, promoters, terminators, and the regions used as probes are indicated. ORF notations and nucleotide (nt) numbers correspond to those of the respective genomic regions of strain COL (RefSeq accession no. NC_002951). (B) Northern blot analysis of σ^B-dependent *asp23* transcription in Newman, SM2 (Δ*spoVG*), and SM99 (*arlR*) grown for 8 h in LB at 37°C. Relevant transcript sizes are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading.

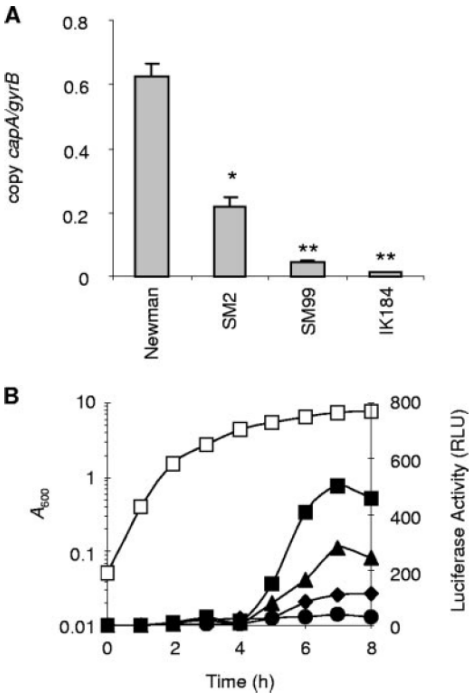

FIG. 6. Expression of *capA* in Newman and its derivatives. (A) Quantitative transcript analysis of *capA* by LightCycler RT-PCR of strains Newman, SM2 (Δ*spoVG*), SM99 (*arlR*), and IK184 (Δ*rsbUVW-sigB*) grown for 8 h at 37°C in LB. Transcripts were quantified in reference to the transcription of gyrase (in numbers of copies per copy of *gyrB*). Values from two separate RNA isolations and two independent RT-PCRs each were used to calculate the mean expression levels (± standard errors of the mean). *, $P < 0.05$ for derivative versus Newman; **, $P < 0.01$ for derivative versus Newman. (B) Growth curve of Newman (open squares) and transcriptional activity of the *capA* promoter in plasmid pSTM04-carrying strains Newman (squares), SM2 (triangles), SM99 (diamonds), and IK184 (circles). *capA* promoter activity was determined by measuring the luciferase activity of the *capAp-luc*$^+$ fusion. Shown are representative results for at least three independent experiments.

while clear 1.1- and 1.0-kb signals were present only in COL and GP268. As for the *arlR* pattern, the 2.9-kb signal was detectable only in the wild-type strains and missing in the Δ*rsbUVW-sigB* mutants. However, the 1.1- and 1.0-kb signals were present in the COL and GP268 Δ*rsbUVW-sigB* derivatives as well, indicating that these transcripts are produced independently from σ^B activity and suggesting that the 1.1-kb signals observed with *arlR* and *arlS* were not identical. Only weak signals were identified with *arlS* in the Newman *rsbUVW-sigB* mutant IK184, and none of the signals were detected in SM99. The impact of σ^B on *arlRS* expression appeared to be strongest in Newman but was also visible in the COL and GP268 backgrounds.

Effect of ArlRS and YabJ/SpoVG on σ^B activity. To assess whether ArlRS and YabJ/SpoVG might have an impact on σ^B activity, we analyzed the transcription of *asp23*, a marker gene for σ^B activity in *S. aureus* (11, 12, 22), during a later growth stage in Newman, SM2, and SM99 (Fig. 5). No difference in *asp23* expression was found between the wild type and its Δ*spoVG* derivative, while the *arlR* mutant showed a slight but reproducible reduction in the expression of the 1.5-kb transcript but not of the 0.7-kb transcript. Interestingly, variations in the expression levels of these two directly σ^B-controlled transcripts were recently observed in a Newman *hemB* mutant, indicating the presence of factors modulating σ^B activity in the recognition of its promoter consensus sequences under certain circumstances (49).

Effect of the *arlRS* locus on *yabJ-spoVG* transcription and vice versa. The inactivation of *arlR* did not affect *yabJ-spoVG* transcription, nor did the deletion of *spoVG* alter *arlRS* expression, suggesting that both loci are independent from each other (data not shown).

Effect of σ^B, ArlRS, and the *yabJ-spoVG* locus on *capA* transcription. Previous studies showed that transcription of the *cap* operon is growth phase dependent and affected by various global regulators in *S. aureus*. Expression of the *cap* operon is predominantly driven by the major promoter located at the beginning of the operon, although several internal promoters with weak activities have been identified in some *cap* gene clusters (36, 37, 46). To confirm the previously observed impact of σ^B and ArlRS on *cap* expression (3, 27, 28, 38) and to see whether the *yabJ-spoVG* locus is involved in *cap* operon regulation, we determined the *capA* expression levels in strains Newman, IK184, SM2, and SM99 by real-time RT-PCR (Fig. 6A). Monitoring *capA* expression after 8 h of growth showed that all three mutations significantly reduced *capA* transcription in *S. aureus*. While inactivation of *rsbUVW-sigB* and *arlR* was associated with a strong reduction in *capA* expression

(approximately 47-fold for *rsbUVW-sigB* and 15-fold for *arlR*; $P < 0.01$), deletion of *spoVG* resulted in a 2- to 3-fold reduction in *capA* transcription ($P < 0.05$), compared with the wild-type level. After 24 h of growth, *capA* transcript levels were drastically reduced and detectable only in Newman, SM2, and SM99, resulting in very low *capA*-to-*gyrB* transcript ratios (0.09 ± 0.03 copies of *capA* per copy of *gyrB* in Newman, 0.09 ± 0.01 copies in SM2, and 0.004 ± 0.0002 copies in SM99), while the *capA* transcription levels in IK184 were found to be below the detection limit.

The expression of the *capA* promoter during growth was monitored with the *capA* promoter-*luc*$^+$ reporter gene fusion plasmid pSTM04 by measuring luciferase activity (Fig. 6B). In line with previous findings (3, 27), luciferase activity values increased in the parental strain Newman in a growth phase-dependent manner, starting at the transition from late-exponential growth phase to stationary phase (i.e., at 4 h), reaching its maximum after 7 h of growth, and declining thereafter. The course of luciferase activity in the Δ*spoVG* mutant SM2 followed in principle that in its parental strain, Newman, although the luciferase activity in SM2 was roughly half as strong as that in Newman at most of the time points monitored. In agreement with the real-time RT-PCR results, only a low level of luciferase activity was detectable in the Δ*rsbUVW-sigB* derivative IK184 at all time points analyzed. A clear reduction in luciferase activity was also found for SM99, albeit not as strong as expected from the real-time RT-PCR results.

CP-5 production in Newman, IK184, SM2, and SM99. To see whether and how the alterations in *capA* expression observed in IK184, SM2, and SM99 had an impact on capsule production, we investigated the capsule formations of Newman and its derivatives after growth in LB for 8 and 24 h by indirect immunofluorescence (Fig. 7, only 8-h data shown). While more than half of the Newman cells (57%) incubated with the CP-5 antibodies produced clear fluorescence signals at both time points analyzed, indicating the presence of CPs in these wild-type cells after 8 and 24 h of growth, this was not the case with either SM2, SM99, or IK184. Only 1% of the Δ*spoVG* cells, 2% of the *arlR*-defective cells, and 1% of the Δ*rsbUVW-sigB* cells emitted detectable amounts of fluorescence under these conditions, suggesting that all three mutants were strongly impaired in their abilities to produce CPs.

***trans*-Complementation of SM2.** In order to evaluate whether the decrease in *capA* transcription in SM2 and its impact on CP-5 formation were due to the inactivation of *yabJ-spoVG*, we constructed plasmid pSTM05, carrying the *yabJ-spoVG* operon, and assessed its impact on *capA* transcription and CP-5 production in the *trans*-complemented mutant. Introduction of pSTM05 into SM2 restored the ability of the *trans*-complemented mutant to produce a capsule (Fig. 7B). While 52% of the SM2 cells harboring plasmid pSTM05 were CY-3 positive after 8 h of growth, only 3% of the SM2 cells transformed with the empty control plasmid pGC2 produced detectable amounts of fluorescence. Similarly, introduction of pSTM05 yielded values for *capA* promoter-driven luciferase activity after 8 h of growth that were comparable to those for the wild type (475 ± 31 relative light units [RLU] for Newman and 510 ± 42 RLU for SM2 harboring pSTM05), while introduction of pGC2 into SM2 had no effect on *capAp*-dependent luciferase activity (246 ± 30 RLU for SM2 and 208 ± 25 RLU for SM2 harboring pGC2). *trans*-Complementation assays performed with SM2 transformed with plasmids harboring either *yabJ* under the control of its σ^B-dependent promoter (pSTM11) or a P_{yabJ}-*spoVG* fusion (pSTM13), on the other hand, failed to revert the effect of the *spoVG* deletion on capsule formation, signaling that both *yabJ* and *spoVG* are required to complement SM2 (data not shown).

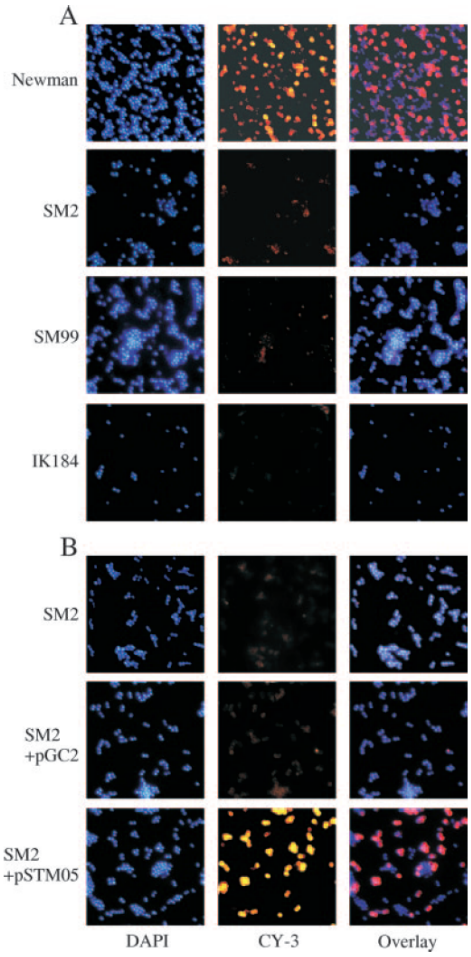

FIG. 7. Capsule production in Newman and its derivatives grown for 8 h in LB at 37°C. (A) CP-5 expression determined by indirect immunofluorescence of strain Newman and its derivatives SM2 (Δ*spoVG*), SM99 (*arlR*), and IK184 (Δ*rsbUVW-sigB*). (B) *trans*-Complementation of Δ*spoVG* mutant SM2. CP-5 expression was determined by indirect immunofluorescence of SM2, and SM2 transformed with either pSTM05 (P_{yabJ}-*yabJ-spoVG*) or the empty control plasmid pGC2. Bacteria were stained with DAPI, marked with CP-5-specific monoclonal antibodies, and stained with Cy3-conjugated anti-mouse antibodies (CY-3).

Two-plasmid testing. The lack of apparent σ^B-specific consensus sequences in the *arlRS* and *capA* promoters suggests the transcription of these operons to be indirectly controlled by σ^B activity. To support this hypothesis, we cloned the promoters of *arlRS* and *capA* into reporter plasmid pSB40N and tested the resulting plasmids pSTM06 (*arlRSp*) and pSTM07 (*capAp*),

respectively, in a heterologous two-plasmid system that was recently shown to be suitable for the identification of σ^B-dependent *S. aureus* promoters (14). Plasmid pSA0455p, harboring the σ^B-dependent promoter upstream of *yabJ-spoVG* (3), which was used as a positive control, and plasmids pSTM06 and pSTM07, harboring the *arlRS* or *capA* promoter, were each transformed into *E. coli* XL1Blue cells containing either pAC7 or pAC7-*sigB* (14), respectively, and the clones obtained were selected on LBACX-ARA plates (43). Transformants containing pAC7 produced uncolored colonies, indicating that none of the introduced promoters were recognized by any form of *E. coli* RNA polymerase holoenzyme. Transformants containing pAC7-*sigB* and pSA0455p were blue on selective LBACX-ARA plates, demonstrating that the activity of the *yabJ-spoVG* promoter was dependent upon arabinose-induced heterologous expression of the *S. aureus sigB* gene in *E. coli* and indicating that the *S. aureus* σ^B-*E. coli* RNA polymerase holoenzyme hybrid was capable of recognizing the heterologous *S. aureus yabJ-spoVG* promoter. In contrast, transformants containing either pAC7-*sigB* and pSTM06 (*arlRSp*) or pAC7-*sigB* and pSTM07 (*capAp*) remained uncolored on selective LBACX-ARA plates, demonstrating that neither the *arlRS* nor the *capA* promoter region was directly recognized by the σ^B-containing RNA polymerase holoenzyme.

Attempts to clone *spoVG* under the control of an inducible promoter. To support our hypothesis that SpoVG might act as regulator downstream of σ^B, we tried to construct a plasmid harboring *spoVG* under the control of an inducible promoter but were not successful with either *E. coli* or *S. aureus*. All our attempts to clone *spoVG* downstream of a σ^B-independent promoter resulted in mutations in the promoter, in the ribosomal binding site, or in the ORF of *spoVG*, signaling that *spoVG* expression needs to be tightly controlled by σ^B or a factor that is dependent on σ^B (data not shown).

DISCUSSION

A recent transcriptional profiling of the σ^B regulon in *S. aureus* indicated the alternative transcription factor to affect the expression levels of 251 genes or operons (3). While most of the genes/operons identified as upregulated by σ^B in that study were also preceded by nucleotide sequences resembling the *S. aureus* σ^B promoter consensus sequence (14), still a significant number of genes/operons found to be upregulated by σ^B lacked such a nucleotide sequence in their promoter regions, including *arlRS* and the *cap* operon (3). Although it is still possible that the latter group of genes/operons might be transcribed by the direct action of a σ^B-containing RNA polymerase holoenzyme, it is more conceivable that σ^B controls the expression of a regulator(s), which would subsequently promote the expressions of these genes/operons. Our Northern analysis performed here confirmed the positive impact of σ^B on *arlRS* and *capA* expression. However, unlike what was suggested by the microarray analyses, the Northern analyses performed here demonstrated *arlRS* expression to be affected by σ^B activity not only in *S. aureus* Newman but in strains COL and GP268 as well. While *arlRS* transcription was found to be highly dependent on σ^B activity in Newman during the later growth stage (8 h of growth), its effect appeared to be less pronounced in COL and the NCTC8325 derivative GP268. Interestingly, our Northern analysis of the *arlRS* locus identified several further transcripts in addition to the 2.7- and 1.5-kb transcripts that were observed in the NCTC8325 derivative RN6390 (10), suggesting that the *arlRS* locus underlies a complex and strain-dependent regulatory circuit. Regulation of *arlRS* by σ^B is likely to be indirect, since the nucleotide sequence preceding *arlR* was not recognized by a two-plasmid system for the identification of promoters recognized by RNA polymerase containing *S. aureus* σ^B (14), leaving open the question of how σ^B affects *arlRS* expression.

Expression of the *cap* operon and capsule formation in *S. aureus* are known to be under multiple levels of control and affected by various environmental stimuli. In line with previous findings indicating σ^B to take part in the control of *cap* operon expression (3, 38), our quantitative RT-PCR results presented here confirmed σ^B to be important for *cap* operon transcription in *S. aureus*, since *capA* expression was drastically reduced in the Δ*rsbUVW-sigB* mutant IK184. Likewise, the *capA* promoter-driven luciferase activity in IK184 remained at a constantly low level throughout growth, in contrast to that in the wild type, where the luciferase activity increased with time, yielding a 20- to 30-fold difference between Newman and IK184 after 8 h of growth. As could be expected from the strong impact of σ^B on *cap* operon transcription, we could show that σ^B is also essential for capsule formation, since IK184, unlike its parental strain, was drastically impaired in its ability to produce CP-5 after 8 and 24 h of growth. Since the two-plasmid testing of the *capA* promoter sequence failed to identify a direct interaction between *capAp* and the σ^B-containing RNA polymerase, its effect on *cap* operon expression is likely to be indirect and mediated via a downstream-acting regulator(s) that is itself controlled by σ^B activity. One such regulator might be ArlR of the ArlRS system, which we confirmed here to be influenced by σ^B activity in *S. aureus*, and which is known to affect *cap* operon transcription and capsule formation (28). However, since our quantitative RT-PCR results for *capA* in *arlR* mutant SM99 yielded a 15-fold reduction in *capA* transcription in comparison with the wild-type level, and since the *capA* promoter-driven luciferase activity in SM99 was only 4- to 5-fold reduced after 8 h of growth, an additional regulator(s) that mediates the effect of σ^B on *cap* operon expression has to be proposed. To test whether the σ^B-dependent SpoVG homolog of *S. aureus* might fulfill such a function, we constructed strain SM2 lacking *spoVG*. We found that the deletion of *spoVG* in *S. aureus* Newman yielded a strong polar effect on *yabJ* transcription, suggesting the Δ*spoVG* mutant SM2 to represent a phenotypic *yabJ-spoVG* double mutant. We found that deletion of *spoVG* decreased *capA* transcription two- to threefold. Interestingly, deletion of *spoVG* strongly repressed the capacity of the mutant to produce CP-5, although SM2 was found to still produce significant amounts of *capA* transcripts. One possible explanation for the discrepancy between *capA* transcription and capsule production observed in SM2 might be an additional influence of YabJ/SpoVG on the expression of internal promoters of the *cap* operon. Alternatively, the *yabJ-spoVG* locus might affect CP production on transcriptional and posttranslational levels, as has been shown for SarA (24). Interestingly, since inactivation of *sarA* is known to increase extracellular protease activity (5) and capsule stability (24), it might be that the negative effect of the *spoVG*

deletion on the extracellular protease activity contributed to the CP-negative phenotype in SM2.

Our data clearly suggest the effector molecules of the *yabJ-spoVG* locus to contribute to the network of regulatory molecules that control *cap* operon transcription and CP-5 formation in *S. aureus* Newman, albeit on a lower level than ArlRS and σ^B. Moreover, the observation that the deletion of *spoVG* influenced extracellular protease production and *capA* transcription and suppressed CP-5 formation indicates that YabJ and/or SpoVG might indeed function as a regulatory molecule in *S. aureus*. This hypothesis is further supported by our findings that deletion of *spoVG* in methicillin-resistant *S. aureus* and in glycopeptide intermediate-resistant *S. aureus* strains significantly reduced the resistance levels against β-lactams and glycopeptides, respectively (S. Meier and M. Bischoff, unpublished results), two phenomena that have also been associated with σ^B activity (1, 32, 41, 50, 58). Considering the impact of *spoVG* deletion on capsule production and on resistance formation in methicillin-resistant *S. aureus* and glycopeptide intermediate-resistant *S. aureus*, and in the face of our recent findings demonstrating *yabJ-spoVG* expression to be highly dependent on σ^B activity (3), we propose the effector molecules of the *yabJ-spoVG* locus of *S. aureus* to act as regulatory molecules that mediate, together with ArlRS, the effect of σ^B on capsule formation.

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